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Functional analysis of the mouse *CBP* gene in the adult central nervous system

by Zuwen Zhang

**Thesis submitted to the Open University for the degree
of Doctor of Philosophy**

August 2001

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献给我所有的家人！

没有他们各方面的全力支持，尤其是在我动荡的生活中总是有一个归处让我安享平静。否则，我实在是难以想象可以顺利完成该论文。

自古道忠孝难以两全。可是我既谈不上忠，又谈不上孝，这又算得是什么呢？有时我也常常纳闷，我为什么要漂泊国外？我到底图得是什么？自九一年在北京求学起十多年来的飘零生活，每当我离开家门，最不敢看的就是妈妈的眼睛，否则我不知还能不能狠心离开，虽然妈妈从没有当面流过泪。这么多年来常伴随着我始终是妈妈那双企盼的眼睛。虽然妈妈身边儿孙绕膝，可是作为一个为子女愿付出一切的普通母亲晚年的唯一愿望：所有子女团聚一堂，安享快乐和平静，我却不能满足，为此我深感内疚。

我谨以此文献上最衷心的祝福，

祝全家和和睦睦，永远平安，快乐！无论何时，我都拥有这个温馨的家可归。

祖文



公元二〇〇一年八月

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Declaration

I declare that no part of this thesis has previously been submitted for a academic degree or other qualification to the Open University or any other university or institution.

There is no collaboration has been made concerning the work in this thesis.

I agree that this thesis, if approved for the Doctoral degree of Philosophy, is deposited in the University Library and maybe available and photocopied at the discretion of the Librarian.

Zuwen Zhang

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Functional analysis of the mouse *CBP* gene in the adult central nervous system

Abstract

CBP (CREB-binding protein) was originally characterized as a transcriptional coactivator of CREB (cAMP response element-binding protein) which is implicated in many processes including the formation of long-term memory. CBP itself is regulated by nuclear Ca^{2+} and Ca^{2+} /Calmodulin kinase IV, suggesting a crucial role in synaptic plasticity. Mutations in human *CBP* gene are associated with the Rubinstein-Taybi syndrome (RTS) characterized by mental retardation and patterning abnormalities. These features suggest that CBP plays a central role in cAMP-mediated gene expression, which in turn is implicated in the formation of long-term memory.

The expression patterns of CBP and its homologue p300 were analyzed. Both are expressed in most of forebrain areas, with particularly high expression in the hippocampus. Parts of the mouse *CBP* gene were characterized. Six exons encode the CREB-binding domain (CBD). Two gene targeting experiments were performed to generate mouse lines with mutations in the *CBP* gene. The first was a deletion of the CBD exon 2, resulting in a frameshift and, thus, a truncated CBP protein ($\text{CBP}^{\text{stop523}}$). In the second experiment, a point mutation was aimed to be introduced in the CBD exon 5 ($\text{CBP}^{\text{Tyr658Ala}}$). After germline transmission, three mouse lines were obtained from $\text{CBP}^{\text{stop523}}$ allele: $\text{CBD2}^{+/-}$, a general deletion of the CBD exon 2; CBD2.floxed , which will be crossed with different Cre mouse lines for tissue-specific mutants; and CBD2.floxed.neo , which might be a hypomorphic allele due to the Neo cassette. For the construct $\text{CBP}^{\text{Tyr658Ala}}$, none of the chimeras gave germline transmission, as apparently

the splicing of the inverted allele was incorrect, possibly leading to strong reduction of vitality. Meantime, six CBP mutants were generated to elucidate functional domains of CBP *in vitro*: a deletion mutation CBP Δ CBD2-5; CBP1100aa, which was reported in mutant mice displaying RTS-like phenotypes; and a point mutation CBP658Ala, which was shown to be critical for interaction with phospho-CREB. The other three mutants retain different sizes of truncated N-terminal CBP and similar proteins were observed in RTS. Both CBP658Ala and CBP Δ CBD2-5 interfered with the cAMP pathway, but not with nuclear receptor-mediated functions. RTS mutants showed strong inhibitory effects on the cAMP pathway.

Abbreviations

AC	adenylyl cyclase
AKAPs	PKA-specific anchoring proteins
AMD	amygdala
AP1	activator protein 1
AP5	a specific antagonist for NMDA receptors
ATFs	activating transcription factors
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BSA	bovine serum albumin
bZIP	basic leucine zipper domain
CA1, 3	hippocampal CA1, 3 regions
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3',5'-monophosphate
CBD	CREB-binding domain
CBP	CREB-binding protein
CH	Cys/His residues-rich region
Ci	cubitus interruptus
CKII	casein kinase II
CM	central medial thalamus
CNS	central nervous system
Cre	Cre recombinase
CRE	cAMP response element
CREB	CRE-binding protein
CREM	CRE modulatory factor

CTP	cytidine triphosphate
CX	cortex
DEPC	diethyl pyrocarbonate
DG	dentate gyrus
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide monophosphate
dpc	days post coitus
DTT	dithiothreitol
E	embryonic days
EDTA	ethylenediaminetetraacetic acid
Ent	entorhinal cortex
ES	embryonic stem cells
F9 EC	F9 embryonal carcinoma cells
FCS	fetal calf serum
Flp	flipase
frt	flipase recognition target
G _i	inhibitory G proteins
G-proteins	GTP-binding proteins
G _s	stimulatory G proteins
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
HA	hemagglutinin

HCG	human chorionic gonadotropin
hh	hedgehog
HIF-1	hypoxia-inducible factor-1
HRP	horseradish peroxidase
IEGs	immediate early genes
IPTG	isopropyl- β -thiogalactopyranoside
IQ	intelligence quotient
IR	ionizing radiation
KID	kinase-inducible domain
KIX	small region for binding to phospho-CREB
LB	Luria broth
LIF	leukemia inhibitory factor
lox66	mutated loxP at right element
lox71	mutated loxP at left element
loxP	locus of crossover in P1 bacteriophage
LTM	long-term memory
LTP	long-term potentiation
MRI	magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
NT	neurotransmitter
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGK-Neo	phosphoglycerate kinase-neomycin phosphotransferase

PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PMSG	pregnant mare serum gonadotropin
PP	phosphatase
RA	retinoic acid
RAR	retinoic acid receptor
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
RTS	Rubinstein-Taybi Syndrome
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
STM	short-term memory
TAD	transactivation domain
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TBP	TATA-box-binding protein
TE	Tris-HCl/EDTA buffer
TF IIA,B..	transcription factor IIA,B...
TGF- β	transforming growth factor- β
tk	thymidine kinase
U	international unit
UV	ultraviolet
WT	wild-type

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Chapter 1

Introduction

1.1 cAMP pathway and memory

1.1.1 cAMP

Cyclic adenosine 3',5'-monophosphate (cAMP) was first identified as an intracellular mediator of hormone action in 1959, and since then has been recognized to act as an intracellular second messenger in many prokaryotic and eukaryotic cells (Sutherland, 1970). In response to extracellular stimuli, the concentration of cAMP (normally $\leq 10^{-7}$ M at physiological level) will be changed up or down: cAMP levels can change five-fold in seconds upon hormonal stimulation (Alberts *et al.*, 1994). cAMP is synthesized from adenosine triphosphate (ATP) by plasma membrane-bound enzymes, adenylyl cyclases, and rapidly destroyed by cAMP phosphodiesterases which hydrolyze cAMP to adenosine 5'-monophosphate (5'-AMP). Many extracellular signaling molecules such as hormones, growth factors, and neurotransmitters may alter the activity of adenylyl cyclase, and thereby control cAMP level. Notably, the alteration of cAMP level mainly depends on the types of target cells (Antoni, 2000).

1.1.2 cAMP and CREB

Intracellular levels of cAMP are regulated primarily by adenylyl cyclase. This enzyme is in turn modulated by various extracellular stimuli mediated by receptors and their interaction with G-proteins (Gilman, 1987; McKnight *et al.*, 1988). The binding of a specific ligand to a receptor results in the activation or inhibition of the cAMP-

dependent pathway. The neuropeptide gene somatostatin was one of the first genes shown to be regulated by cAMP (Montminy *et al.*, 1986a). Deletion studies of the 5'-upstream region of the somatostatin gene identified the cis-acting element TGACGTCA, an 8 bp palindromic sequence, named a cAMP response element (CRE), which was later found in the promoter region of many other target genes (Montminy *et al.*, 1986b). A comparison of the CRE sequences identified showed that the 5'-terminus of the palindromic sequence, TGACG, is more conserved, whereas the 3'-terminus, TCA motif, is less constant (Sassone-Corsi, 1995). The binding site specificity appears to require 18-20 bp, since 5 bases flanking the core consensus have been shown to dictate the permissivity of transcriptional activation (Deutsch *et al.*, 1988). The search for CRE motif-recognizing factors led to the identification of CREB (cAMP response element-binding protein), a transcription factor belonging to the family of basic leucine zipper transcription factors (Gonzalez *et al.*, 1989b).

cAMP is also a central molecule in synaptic plasticity and memory consolidation. During learning, extracellular stimuli such as neurotransmitters activate cell membrane receptors (G protein-coupled receptors), which in turn activate heterotrimeric GTP-binding proteins (G-proteins). Certain receptors activate adenylyl cyclase through stimulatory G protein (G_s), while others inhibit it through inhibitory G protein (G_i). In its inactive form, G_s exists as a trimer of G_α , G_β and G_γ with G_α bound to GDP. After receptor activation, G_s dissociates to G_α and $G_{\beta\gamma}$ subunits. The G_α activates adenylyl cyclase, while $G_{\beta\gamma}$ can stimulate other pathways. After G_α has hydrolyzed GTP, it no longer stimulates adenylyl cyclase, and binds again as a tight complex to $G_{\beta\gamma}$.

The dissociated G-protein units influence the activity of various effector molecules including adenylyl cyclase. This enzyme then increases the intracellular cAMP concentration leading to the activation of cAMP-dependent protein kinase A (PKA) in

the cytoplasm and to the modification of ion channels and synaptic proteins. The PKA holoenzyme, which consists of two catalytic subunits and two regulatory subunits, is localized in the cytoplasm, presumably by the interaction of the regulatory subunit R2 with specific anchoring proteins called AKAPs (Faux and Scott, 1996). If the extracellular stimuli are persistent for a critical period of time, binding of cAMP to the regulatory subunits causes the release of the catalytic subunits of PKA, which are then free to translocate to the nucleus from cytoplasmic and Golgi complex anchoring sites to phosphorylate a number of substrates e.g., transcription factors on serines in the context X-Arg-Arg-X-Ser-X (Roesler *et al.*, 1988; Meinkoth *et al.*, 1990).

PKA has to phosphorylate the transcription factor CREB protein at serine-133 to achieve memory consolidation (Gonzalez and Montminy, 1989a). Phospho-CREB then binds as a homodimer to CREs, thus, activates the transcription of immediate early genes (IEGs). The protein products of many IEGs are themselves transcription factors that can activate the transcription of late response genes, which are likely to encode proteins necessary for long-term modulation of synaptic function (Fig. 1.1). Nuclear localization of phosphatases, named PP1 and PP2-A, are responsible for CREB dephosphorylation. PP-1 is present in the nucleus, while PP2-A is located mainly in the cytoplasm (Hagiwara *et al.*, 1992; Wadzinski *et al.*, 1993; Bito *et al.*, 1996).

1.1.3 cAMP and LTP

It has been shown that CRE-mediated gene expression is stimulated by signals that induce long-term potentiation (LTP) (Impey *et al.*, 1996). LTP is defined as a long-lasting increase of synaptic efficacy following a brief high-frequency stimulation. LTP was discovered by Bliss and Lomo (1973) in the dentate gyrus and since then has been studied widely as a promising cellular mechanism for learning and memory. In the

hippocampus, synaptic potentiation can be classified according to its responsive duration. Short-term potentiation (STP) presumably lasts for minutes, in contrast, LTP lasts at least 1 hour and may last up to several days.

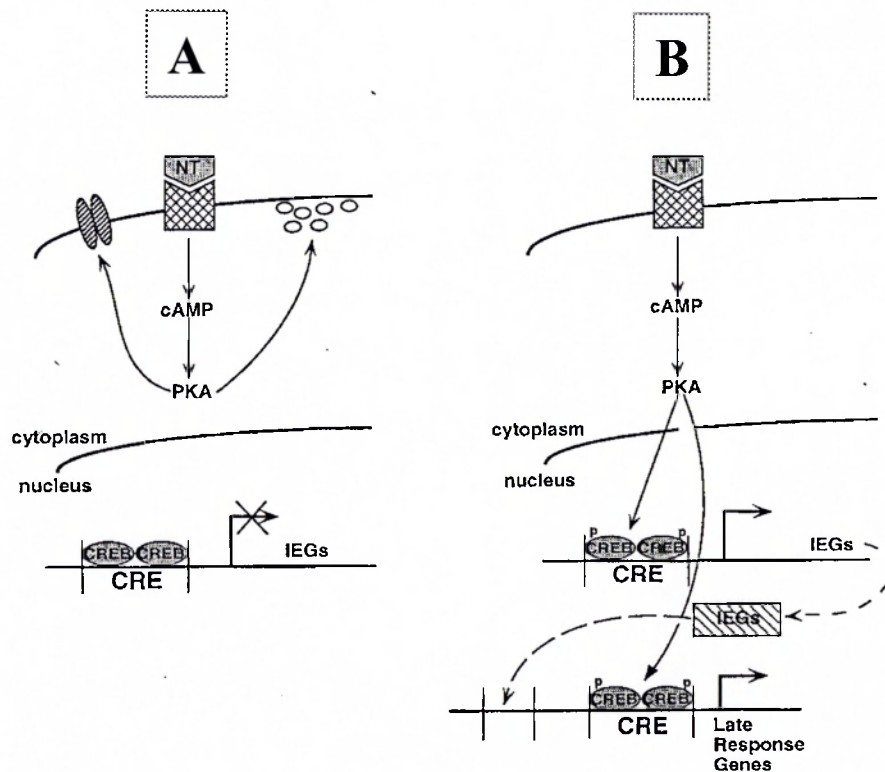


Fig. 1.1: Mechanisms of cAMP pathway and formation of long-term memory (LTM) (A). Short-term exposure to a neurotransmitter leads to the brief generation of cAMP that can activate PKA, and can lead to the modification of ion channels and synaptic proteins. (B). Long-term exposure to a neurotransmitter leads to continuous increase of cAMP, allowing the catalytic subunits of PKA to translocate to the nucleus to phosphorylate CREB, thereby to activate transcription of IEGs. Many IEG proteins are themselves transcription factors that activate the late response gene transcription. Those proteins are likely required for the formation of LTM.

A systematic study of the distribution of synapses that possesses the ability to undergo LTP in the limbic system was carried out. It was shown that the lateral olfactory tract

shows no LTP, the pyriform cortex shows little LTP, followed by the amygdala, entorhinal cortex, perforant path, hippocampal CA1, septal area and fornix/fimbria (Racine *et al.*, 1983). Note that hippocampal pathways show significantly larger LTP effects than do the non-hippocampal pathways. LTP has been observed in the three major excitatory synapses in the trisynaptic circuit of the hippocampus (Fig. 1.2).

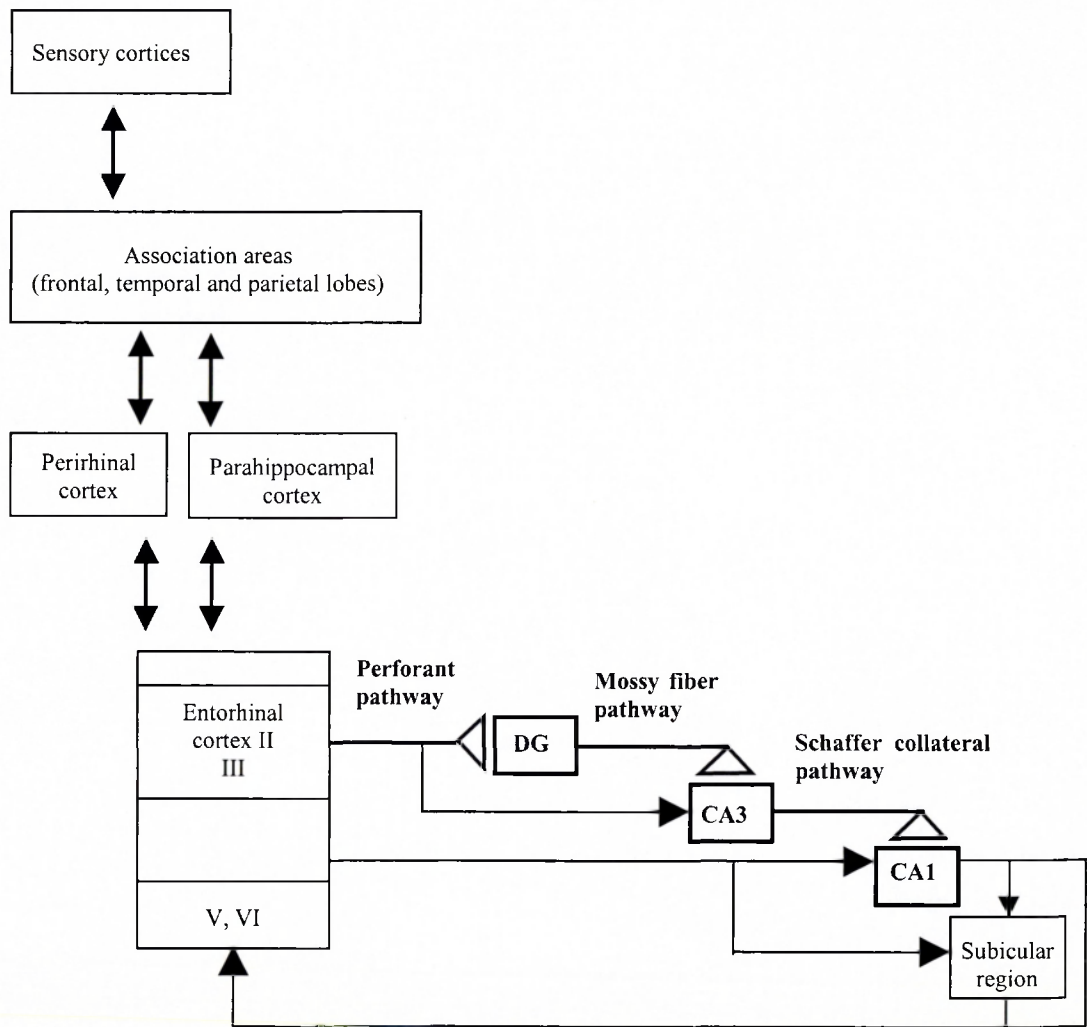


Fig. 1.2: Three major excitatory synapses in the trisynaptic circuit of the hippocampus: perforant pathway, mossy fiber pathway and Schaffer collateral pathway.

The perforant pathway starts from the pyramidal cells of the entorhinal area to the granule cells of the dentate gyrus (dentate LTP); the mossy fiber pathway starts from the granule cells of the dentate gyrus to the CA3 pyramidal cells (mossy fiber LTP); and

the Schaffer collateral pathway starts from the CA3 pyramidal cells to the CA1 pyramidal cells (CA1 LTP). Both dentate LTP and CA1 LTP are initiated postsynaptically by the activation of N-methyl-D-aspartate (NMDA) receptors. In contrast, CA3 LTP is independent of NMDA receptors and is thought to be initiated presynaptically. It was shown that LTP of the hippocampal slice in either the dentate gyrus following perforant path stimulation or the hippocampal CA1 area following Schaffer collateral stimulation, resulted in an increase in the incorporation of labelled valine into proteins destined for secretion into the extracellular medium from just those regions that had been stimulated (Bennett *et al.*, 2000). Moreover, induction of LTP was also found following the increase in the size of dendritic spines (Fifkova *et al.*, 1982) and the amount of neurotransmitters released (Malinow and Tsien, 1990).

The hippocampus is a major component of the medial temporal lobe, a brain system that plays an important role in declarative or relational memory (Eichenbaum *et al.*, 1992; Squire 1992). Synaptic plasticity at the trisynaptic circuit of the hippocampus has been well defined anatomically and best studied in relation to learning and memory (see Fig. 1.2). Lesion studies in rodents showed that two forms of declarative memory, spatial memory and contextual fear memory, depend on the intact hippocampus (Morris *et al.*, 1982; Kim and Fanselow 1992; Phillips and LeDoux 1992; Chen *et al.*, 1996). Furthermore, both spatial memory and contextual fear memory have been shown to be disrupted by AP5, a specific antagonist for NMDA receptors. Mutant mice containing a CA1-specific inactivation of the NMDA receptor 1 showed a complete loss of LTP in hippocampal CA1 region as well as a profound defect in spatial memory (Tsien *et al.*, 1996). These results support the notion that LTP in the Schaffer collateral pathways is important for memory formation, and that the formation of LTP requiring the activation of NMDA receptors is critical for those hippocampus-dependent learning and memory

(Swanson *et al.*, 1982; McNaughton *et al.*, 1986). The initiating step for LTP in Schaffer collateral pathway involves the release of glutamate from the presynaptic terminals of the CA3 neurons, which leads to the activation of NMDA receptors on the postsynaptic CA1 pyramidal cells, resulting in an influx of Ca^{2+} into the postsynaptic neuron. The Ca^{2+} signal, in turn, activates many gene transcription through a number of second messenger kinases, including protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and many tyrosine kinases.

1.2 Transcription factor CREB

1.2.1 CREB family

CREB belongs to the large family of leucine zipper CREB/ATF transcription factors (Gonzalez *et al.*, 1989b). The mammalian proteins show a high degree of homology throughout their C-terminal regions, which contain the bZip domain, but outside of this region, sequence homology between the family members is relatively poor. In rodents, the CREB/ATF family is comprised of at least ten genes (Brindle and Montminy, 1992), in addition to CREB, including CREM (CRE modulatory factor) and its variants, activating transcription factor (ATF1, ATF2, ATF3, and ATF4). All of these transcription factors can bind to the CRE sequence as homodimer or heterodimer, leading to extensive cross-interactions. Many of these genes are rapidly activated in response to the elevation of cytoplasmic cAMP or Ca^{2+} levels. Furthermore, each of the genes is thought to encode many isoforms by different mechanisms, such as alternative splicing, use of alternative initiation codons and/or an alternative intronic promoter (Sassone-Corsi *et al.*, 1995). Among the isoforms, some proteins activate gene

transcription, and some repress. For example, partial or complete deletions of glutamine-rich regions give rise to repressors, e.g. CREB2b in *Drosophila*, and CREM α , CREM β and CREM γ in mammals (Yin *et al.*, 1995). In *Aplysia*, the insertion of premature stop codons in the *CREB* gene produces truncated proteins that lack the DNA-binding domain and the nuclear localization signal (Bartsch *et al.*, 1998). Different members of the CREB/ATF superfamily also function differently on many signal transduction pathways. Within the brain, CREB subtype factors are the major CRE activator, while CREM factors may function primarily as negative modulator of CREB (Pham *et al.*, 1999).

1.2.2 Structure of CREB

CREB binds to CRE sites on target genes as a homodimer formed via the leucine zipper motif present at its C-terminus. In the last decade, numerous studies have contributed much to our understanding of CREB structure and function, and CREB-mediated gene transcription. CREB exists in three alternatively spliced isoforms (Ruppert *et al.*, 1992; Blendy *et al.*, 1996), CREB α , CREB β and CREB δ , which have different developmental regulation (Walton and Dragunow, 2000). Most of CREB/ATF proteins consist of three functional domains (Montminy, 1997): the basic/leucine zipper (bZIP) domain which mediates dimerization and DNA-binding properties and is necessary for translocation of CREB into the nucleus (Johnson and McKnight, 1989; Waeber and Habener, 1991); the transactivation domain (glutamine-rich domain Q1 and Q2) are important for basal transcription (Q1) and interaction with the TATA-box-binding protein (TBP)-associated factor hTAF₁₃₀ (Q2), a component of TFIID complex (Quinn, 1993; Xing and Quinn, 1994; Ferreri *et al.*, 1994); and the kinase-inducible domain (KID) contains the important phosphorylation sites, i.e., Ser-133 for PKA (Lamprecht, 1999), and sites for

other kinases: Ser-115 and -129 for glycogen synthase kinase-3 GSK-3 (Fiol *et al.*, 1994); Ser-133 and -142 for Ca^{2+} /calmodulin dependent kinase II and IV (CaMKII and IV) (Dash *et al.*, 1991; Sun *et al.*, 1994); and Ser-108, -111, -114 for casein kinase II (Saeki *et al.*, 1999) (Fig. 1.3).

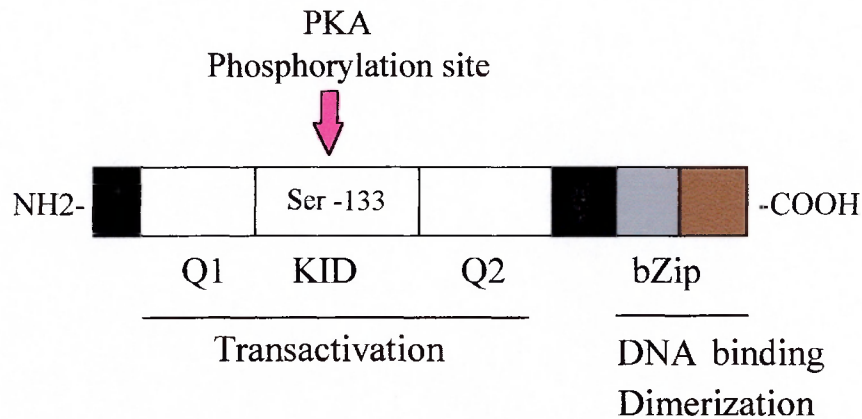


Fig. 1.3: Structure of CREB. The glutamine-rich domain Q1 and Q2, kinase-inducible domain (KID) and basic domain and leucine zipper are shown. The amino acid Ser-133 phosphorylated by cAMP-dependent protein kinase A (PKA) is also indicated.

1.2.3 CREB and LTM

One of the hallmarks of memory consolidation in both vertebrates and invertebrates is the requirement for new protein synthesis. Duffy *et al.* (1981) found that two specific glial proteins are rapidly labelled and released into the extracellular fluid after the goldfish acquired a new pattern of behavior. Long-term but not short-term facilitation of the synaptic connections between neurons requires new mRNA expression and new protein synthesis, and such altered expression level of specific proteins is regulated through the cAMP second messenger pathway. The transcriptional activation of CREB is crucially dependent on phosphorylation of Ser-133 site by cAMP-dependent PKA and Ca^{2+} -dependent calmodulin kinases. It has been suggested that this modification induces a conformational change that transforms CREB from an inactive to an active form (Bito

et al., 1996). Interestingly, in its non-phosphorylated form, CREB can suppress activator protein 1 (AP1) activity by competing with Jun protein for the AP1 site in target genes. As phosphorylated CREB lacks this ability, it acts as a regulator to facilitate transcriptional activation of immediate early genes (Lamph *et al.*, 1990; Masquillier and Sassone-Corsi, 1992).

CREB is proposed to be a key factor that mediates memory consolidation, as both mice homozygous for a hypomorphic allele of the *CREB* gene (Bourtchuladze *et al.*, 1994; Blendy *et al.*, 1996) and *Drosophila* overexpressing a dominant-negative form of CREB show a reduced ability to form long-term memory (Yin *et al.*, 1994; 1995). Mice with a targeted disruption of the α and Δ isoforms of CREB are profoundly deficient in long-term memory based on the behaviour tests, fear conditioning and Morris water maze. In contrast, short-term memory, lasting between 30 and 60 minutes, remains normal. Electrophysiological studies in hippocampal slices from these mutants revealed that LTP in mutants is small and decays to baseline within 1.5 hours after induction (Bourtchuladze *et al.*, 1994; Kogan *et al.*, 1997). However, these results have recently been contradicted, as the homozygous mutants for CREB α and CREB Δ have showed no significant abnormalities observed in contextual or cued fear conditioning, or in hippocampal LTP (Gass *et al.*, 1998). Meantime, the third isoform CREB β was strongly up-regulated. CREB β has nearly the same transactivation potential as the other CREB isoforms and is expressed ubiquitously; and transgenic mice expressing a dominant-negative form of CREB (CREB_{A133}) in the limbic system displayed normal LTP and depotentiation in basolateral amygdala (Rammes *et al.*, 2000). These recent results suggest that CREB activity *per se* is dispensable and compensated by other CREB-like proteins.

In *Drosophila*, a gene for CREB (dCREB2) was also cloned, and its two isoforms, activator dCREB2-a and repressor dCREB2-b, were analyzed for a functional role in memory consolidation. The dCREB2-a isoform is a PKA-responsive transcription activator, whereas the dCREB2-b product blocks PKA-responsive transcription induced by dCREB2-a in cell culture. To investigate the role of CREB in LTM formation in *Drosophila*, a transgene that expresses dominant-negative dCREB2-b under the control of a heat-shock promoter (*hs-dCREB2-b*) was generated. Induction of the transgene by heat shock prior to a massed training session did not affect short-term anesthesia-resistant memory. By contrast, induction prior to spaced training severely disrupted long-term memory. Heat shock alone had no effect on memory in wild-type flies or in flies containing a control transgene. They found that the expression of a dominant-negative CREB (dCREB2-b) completely and specifically blocked long-term memory, while the expression of dCREB2-a enhanced long-term memory formation. Mutating two amino acids in the leucine zipper dimerization domain of the CREB repressor was sufficient to prevent the dominant-negative effect on LTM. Thus, induction of LTM is not only protein synthesis-dependent but also CREB-dependent (Yin *et al.*, 1994; 1995). Taken together, these findings suggest that CREB is necessary for the establishment of long-term but not short-term memory in *Drosophila*.

The switch from short- to long-term facilitation induced by behavioral sensitization in *Aplysia* involves CREB-like proteins, as the formation of long-term facilitation in *Aplysia* can be disrupted by drugs that interfere with transcription and translation, and it involves cAMP-dependent gene expression (Dash *et al.*, 1996). Dendritic spines, the loci of excitatory interaction between central neurons, are prime candidates for long-term synaptic modifications. *In vitro* studies with primary cultured rat hippocampal neurons have shown that estradiol induces two-fold the density of dendritic spines and

increases concomitantly the levels of phosphorylated CREB and its major cofactor CREB-binding protein (CBP) (Murphy and Segal, 1997). Blockade of PKA eliminates estradiol-evoked spine formation, as well as the CREB and CBP responses. The dendritic outgrowth can also be inhibited by antisense oligonucleotides specific against CREB. These results indicate that CREB phosphorylation is a necessary step in the process leading to generation of new dendritic spines.

1.3 Transcriptional cofactor CREB-binding protein (CBP)

1.3.1 Characterization of CBP as a cofactor of CREB

As examined by affinity selection assay, phospho-CREB did not appear to associate directly with factors of the basal transcriptional machinery, i.e. TFIIA, B, D, E, F, H or RNA polymerase II. However, the glutamine-rich domain Q2 of CREB interacts with hTAF₁₃₀, a component of the TFIID complex. It was supposed that CREB needs cofactors to interact with the basal transcriptional machinery to further regulate gene transcription. Chrivia *et al.* (1993) screened a human thyroid λ gt11 cDNA library using a recombinant CREB protein labeled at the PKA phosphorylation site with ³²P. Through this binding assay, a portion of a 265 kDa protein was identified, named CREB-binding protein (CBP). Immunohistochemical studies in vertebrate cells demonstrated that CBP appears to be located exclusively in the nucleus. Far Western blot analysis of HeLa nuclear extracts with ³²P-labeled phospho-CREB probe also revealed a single band of 265 kDa, which associated only with phospho-CREB (Arias *et al.*, 1994). *In vitro* binding experiments showed that CBP specifically binds to the Ser-133 phosphorylated kinase-inducible domain (KID) of CREB through its CREB-binding domain (CBD) (amino acid 461-661), which is highly conserved in CBP homologs from

Caenorhabditis elegans, *Drosophila melanogaster* to mammals. Hence, it is likely that a small region termed KIX (amino acid 591- 661) is actually sufficient for binding to phospho-CREB (Parker *et al.*, 1996). α helices formed in the CBD were shown to be crucial for the binding to phospho-CREB. Several hydrophobic residues were found in CBD, e.g., the phosphorylated Ser-133 was shown to form a hydrogen bond with the hydroxyl group of Tyr658 in CBD region. The KID:KIX complex is disrupted by low concentrations of non-ionic detergents, and mutation of hydrophobic residues in either KID or KIX severely affects the binding, suggesting that hydrophobic interactions play an important role in the complex formation (Radhakrishnan *et al.*, 1997). In addition to CREB, the KIX domain of CBP also recognizes the transactivation domains of other nuclear factors, including c-Jun (Arias *et al.*, 1994), c-Myb (Dai *et al.*, 1996), Cubitus interruptus (Akimaru *et al.*, 1997a), Gli-3 (Dai *et al.*, 1999), tumor suppressor gene product BRCA1 (Pao *et al.*, 2000), the E2 protein of human papillomavirus (HPV E2) (Lee *et al.*, 2000), HTLV-1 virally encoded Tax protein (Kwok *et al.*, 1996) and the mouse clock transcription factor Arnt3 (mArnt3, also termed as BMAL1 or MOP3) (Takahata *et al.*, 2000).

1.3.2 Domain structure of CBP

CBP, itself, is also directly regulated by nuclear Ca^{2+} and Ca^{2+} /calmodulin kinase IV (CaMK IV) (Chawla *et al.*, 1998). Moreover, CBP serves also as a coactivator for other transcription factors such as nuclear hormone receptors (Kamei *et al.*, 1996; Hanstein *et al.*, 1996; Chakravarti *et al.*, 1996 and Torchia *et al.*, 1997), p53 (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997), NF- κ B (Perkins *et al.*, 1997; Gerritsen *et al.*, 1997), c-Jun and c-Fos (Bannister *et al.*, 1995a; 1995b), for transcriptional coactivators such as SRC1 (Chen and Li, 1998; Sheppard *et al.*, 2001), and for kinases such as

pp90rsk (Nakajima *et al.*, 1996) and CDK2 (for more details about interacting proteins of CBP see reviews by Janknecht and Hunter, 1996; Goodman and Smolik, 2000). Two small domains directly interacting with phosphorylated CREB range from amino acid residues 597 to 614 and from 647 to 662, which are located on two different exons (CBD exon 3 and exon 5) in the CREB-binding domain (CBD), and tyrosine 658 in CBD exon 5 is shown to be absolutely required for the interaction with phospho-CREB (Fig. 1.4), as a tyrosine to alanine mutation at position 658 fully abolished *in vitro* binding of phospho-CREB with CBP (Radhakrishnan *et al.*, 1997). By mutational analyses of CBP, the interaction domains in CBP with these various factors were characterized (Fig. 1.5).

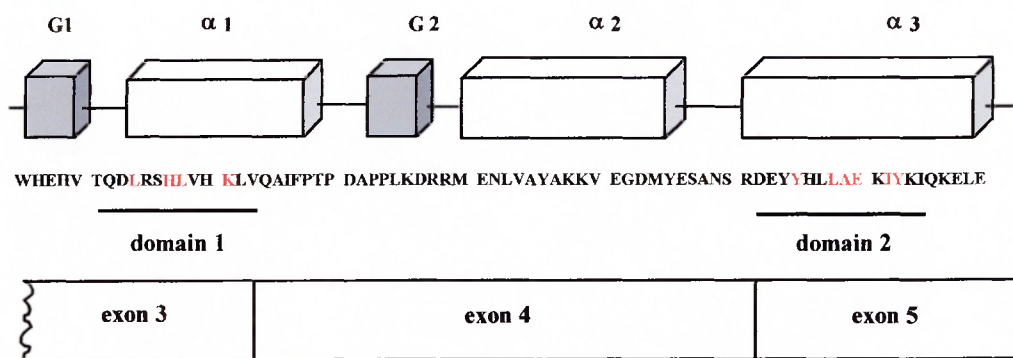


Fig. 1.4: Two small domains of mouse CBP encoded by CREB-binding domain (CBD) exon 3 and 5 are known to bind to phosphorylated CREB. The amino acid residues 591-665 are listed under the corresponding helical structure of CBP; Amino acid residues involved in the binding to CREB are indicated in red.

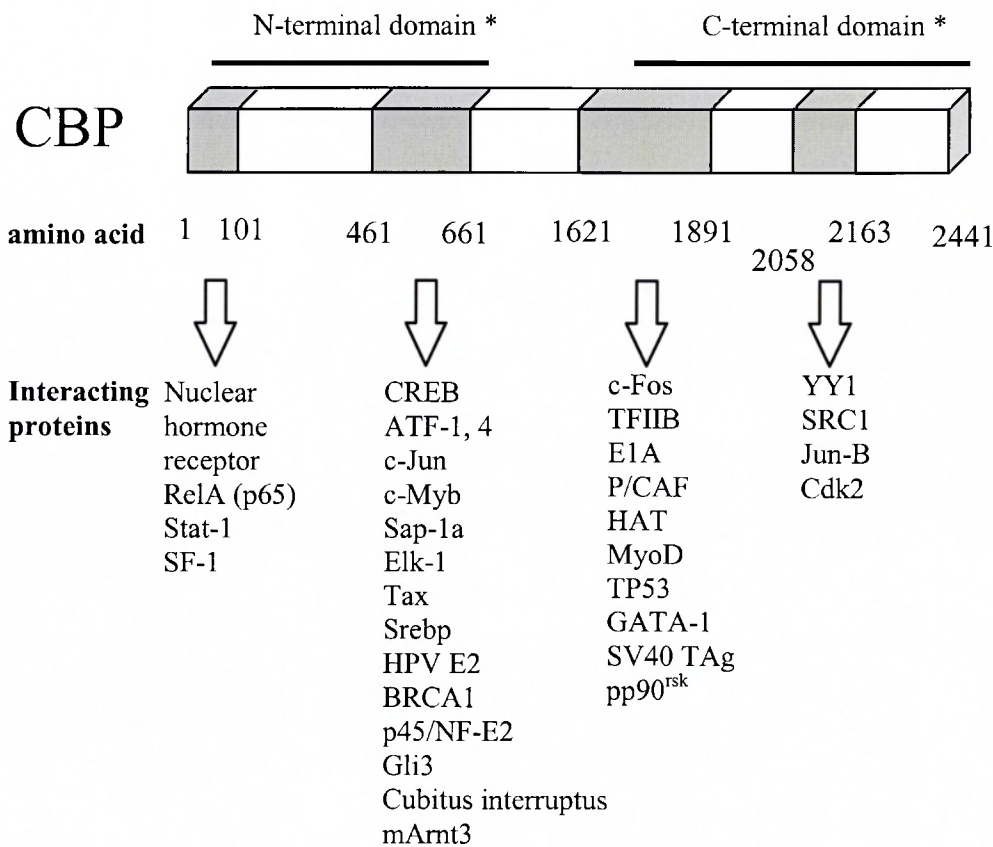


Fig. 1.5: Schematic representation of mouse CBP domains. Amino acid 1-101, 461-661, 1621-1891 and 2058-2163 containing binding sites for the proteins are indicated. There are many other interacting proteins not listed because of space limitation, e.g. NF- κ B and Ets-1 interact with both N- and C-terminal domains; N-terminal domain interacting proteins: RXR, Pit1, Stat-2, HIF-1, TBP, Mdm2, p73, TAL1 and HNF4; C-terminal domain interacting proteins: p53, p/CIP, Smad, Neuro D, C/EBP β , RNA helicase A, E2F, SF-1, Tat, CIITA, HPV E6, Py LT, dMad, JMY, Twist and ATF-2.

1.3.3 CBP and its homologous protein p300

CBP was originally characterized as a co-activator of CREB, while p300, which is highly homologous to CBP (a 2414 amino acid residues for human p300), was first identified as a protein interacting with the adenovirus E1A oncoprotein (Yee *et al*, 1985; Arany *et al*, 1994; Eckner *et al*, 1994), and it is thought to participate in the prevention of the G₀/G₁ transition during the cell cycle, in activation of certain

enhancers and in the stimulation of differentiation pathways (Wang *et al.*, 1993). E1A is believed to mediate transformation and immortalization through two conserved regions, named CR1 and CR2. CR1 is responsible for binding and presumably inhibition of p300 and CBP activity (Moran, 1993; Wang *et al.*, 1993; Smits *et al.*, 1996). Overexpression of CBP and p300 can overcome the E1A-mediated gene repression and block the transformation process, it suggested that E1A represses genes that inhibit cell proliferation by competing for a limiting amount of p300 and CBP. In agreement with this notion, p300 mutations were identified in certain cancers (Muraoka *et al.*, 1996), suggesting that p300 might also function as a tumor suppressor gene.

Several protein motifs in p300 and CBP such as the bromodomain, the KIX domain, and three Cys/His residues-rich (C/H domains) region are well conserved in species ranging from *C. elegans*, *Drosophila* to mammals (Parker *et al.*, 1996; Akimaru *et al.*, 1997b). These domains serve as binding sites for sequence-specific transcription factors and other components regulating gene expression. For example, mediated by one of the conserved C/H domains, p300 and CBP can interact with RNA helicase A, which in turn binds RNA polymerase II (Nakajima *et al.*, 1997a). Recruitment of the RNA polymerase II holoenzyme by p300 and CBP appears to be an essential step in the process of transcriptional activation by CREB (Nakajima *et al.*, 1997b). In mammal, CBP and p300 are 63% identical at the protein level. Greater similarity (85-90% homology) is observed in specific regions of CBP and p300, including the CREB-binding domain (CBD), the E1A binding site and the bromodomain (Arany *et al.*, 1994). 60-70% homology to mammal is observed in the same regions in *Caenorhabditis elegans*, and *Drosophila* homologue of CBP (dCBP) has also been identified and shown to have striking homology with mouse CBP (Akimaru *et al.*, 1997b). Recently, p300/CBP-like polypeptides were identified in the plant *Arabidopsis thaliana*.

Interestingly, homology between animal and plant p300/CBP is largely restricted to a C-terminus, about 600 amino acids in length, which encompasses acetyltransferase and E1A-binding domains (Bordoli *et al.*, 2001).

1.3.4 Functions of CBP and p300

Both CBP and p300 contain many common structural motifs, are highly related in primary structure and functionally overlap to a great extent, since they bind a similar set of cellular target factors (Janknecht and Hunter, 1996) (Fig. 1.5). Accumulating evidence now suggests that both proteins have very similar cellular functions, but also many functional differences have recently been reported (Kawasaki *et al.*, 1998; Yuan *et al.*, 1999; Ugai *et al.*, 1999).

CBP and p300 display pleiotropic functions, several mechanisms by which CBP and p300 contribute to their transcriptional regulation. First of all, CBP and p300 participate in a variety of cellular processes, such as cell growth, proliferation, homeostasis, viral pathogenesis, DNA repair and apoptosis by interaction with multiple transcription factors, indicating that both of CBP and p300 function as molecular integrators in complex signal transduction pathways. CBP and p300 can synergize with each other to interact with many nuclear factors when bound to the same promoter in *cis* position, while inhibition between the factors may occur when CBP and p300 are bound to different promoters. It has been proposed that this results from factor competition for limiting amounts of CBP and p300 in the nucleus (Kamei *et al.*, 1996). For example, since p53 and AP-1 interact with the same domain of CBP and p300, these factors compete with each other functionally.

CBP and p300 directly contact components of the general transcriptional machinery, the transcription factors TFIIB, TFIID, and RNA helicase A. The helicase is proposed to

bridge CBP and p300 and the RNA polymerase II holoenzyme (Nakajima *et al.*, 1997a). For example, as a prerequisite to activate the basal transcriptional machinery, phospho-CREB has to bind to CBP to further activate CREB-mediated gene transcription (Kwok *et al.*, 1994). The adenovirus E1A oncoprotein may compete for the RNA helicase A binding site on CBP, thereby preventing CBP from interacting with RNA polymerase II. The E1A binding to CBP/ p300 may also dissociate CBP and p300 from phospho-CREB. Thus, CBP and p300 are considered as physical bridging molecules between upstream DNA-bound transcription factors and the basal transcriptional machinery.

CBP and p300 have also been shown to have intrinsic histone acetyltransferase (HAT) activity, which decreases the association of the positively charged histones with negatively charged DNA. CBP and p300 acetylate the amino terminal tails of nucleosomal histones, then destabilizes histone-DNA interactions and facilitates access and binding of the DNA to transcription factors. Moreover, CBP and p300 associate with other nuclear cofactors that have acetyltransferase activities, such as p/CAF (p300/CBP-associated factors). Thus, both CBP and p300, in part, facilitate transcription by directly modifying chromatin structure.

Despite the fact that CBP and p300 are structurally similar, their functions are not entirely overlapping. Mutations in one copy of the *CBP* gene cause serious defects in human (Petrij *et al.*, 1995), while p300 does not seem to give a similar syndrome. Kawasaki *et al.* (1998) generated p300 and CBP knock-down cell lines by using specific hammerhead ribozymes. p300 knock-down cell line showed a resistance against retinoic acid-induced differentiation, but CBP knock-down cell lines did not. It has also been shown that both CBP and p300 proteins play different roles in the cellular response to DNA damage (e.g. ionizing radiation, IR). p300, but not CBP, contributes to

IR sensitivity of cells, and IR-induced apoptosis is impaired in the p300-, but not CBP-deficient cells.

1.3.5 Mice with mutations in *CBP* and *p300*

The functions of CBP and its closely related protein p300 have recently been investigated by gene targeting in mouse. Three different alleles of CBP have been generated. First, using conventional knock-out technology, amino acids 29-265 were deleted and replaced by the *neo* cassette (Tanaka *et al.*, 1997), presumably leading to a complete deletion of the CBP protein. Second, using a retroviral gene trap vector, an insertional mutation into the *CBP* gene was generated, leading to a truncated N-terminal CBP (residues 1-1084) as shown by Western blot analysis (Oike *et al.*, 1999a). Third, the mouse *CBP* gene was targeted by homologous recombination in which exons encoding the first Cys/His-rich (CH1) domain (amino acids 340-443) were deleted and replaced by the *neo* cassette. It was investigated whether the mutant *CBP* locus generates a truncated N-terminal CBP (residues 1-340) by Western blot analysis, but none was found (Kung *et al.*, 2000). The mouse p300 allele was also inactivated in which exons encoding the CH1 domain were replaced with the *neo* cassette by homologous recombination (Yao *et al.*, 1998).

The functions of CBP *in vivo* was firstly investigated in the mice heterozygous for *CBP* generated by replacement of amino acid 29-265 with *neo* cassette (Tanaka *et al.*, 1997). *CBP*^{+/-} in both BALB/c and C57BL/6 genetic background displayed abnormal skeletal patterning and severe growth retardation, but the significantly lower frequency of the different abnormalities was found in C57BL/6 background. Similar abnormalities were observed in the human Rubinstein-Taybi Syndrome (RTS), a haplo-insufficiency disorder that is characterized by mental retardation, severe developmental defects and

patterning abnormalities and that is shown to contain mutations in the human *CBP* gene (Petrij *et al.*, 1995) (see below, 1.3.6). The most pronounced phenotype in the $CBP^{+/-}$ mice was delayed ossification of frontal bones with an enlarged anterior fontanel (14/21); various anomalies of the sternum (extra, fusion, reduction, and asymmetry of the ossification with split in the sternum) were found in one-third (7/21) of the heterozygotes. Asymmetric sternocostal joints were also found in the heterozygotes with similar penetrance; an additional phenotype found in the heterozygotes was the malformation of the xiphoid process and vertebrae (6/21). The expression level of bone morphogenetic protein 7 (*Bmp7*) was shown to be significantly reduced in the *CBP* heterozygous mutants. As reported recently, the *Drosophila* homologue of CBP (*dCBP*) functions as a coactivator of the products of the segment polarity gene *cubitus interruptus* (*ci*) (Akimaru *et al.*, 1997a) and *dorsal*, a *Drosophila* homologue of the vertebrate NF- κ B (Akimaru *et al.*, 1997b). *Ci* is a critical transcription factor for pattern formation in the *hedgehog* (*hh*) pathway (Domínguez *et al.*, 1996). In response to Hh, *Ci* activates the *decapentapleigic* (*dpp*) and *wingless* (*wg*) genes, which encode the *Drosophila* homologue of transforming growth factor β /bone morphogenetic protein (TGF- β /BMP) and the Wnt family proteins, respectively. *Dorsal* activates *twist* gene expression, which is critical for dorso-ventral polarity (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991). Taken together, this suggests that CBP contributes to the conserved *dpp/Bmp* signaling pathway in both *Drosophila* and mouse. This could be the explanation for some phenotypes observed in RTS by haplo-insufficiency of *CBP*. Furthermore, analysis of the homozygous *CBP* mutant mice (75% BALB/c, 12.5% C57BL/6 and 12.5% CBA) (Tanaka *et al.*, 2000) showed embryonic lethality at embryonic days (E) 10.5 to 12.5 as a result of massive hemorrhage caused by a defect in blood vessel formation in the central nervous system. In addition, $CBP^{-/-}$ mutants

showed developmental retardation, delays in both primitive and definitive hematopoiesis, open neural tube and defects in mesenchymal cells (Tanaka *et al.*, 2000).

A second mouse model of RTS was generated by an insertional mutation into the *CBP* gene (Oike *et al.*, 1999a). The heterozygous mice (C57BL/6), which had a truncated CBP protein (residue 1-1084) containing the CREB-binding domain, showed clinical features of RTS, such as growth retardation (100%, in 100 mice), retarded osseous maturation (100%), large anterior fontanel (100%), hypoplastic maxilla with narrow palate (100%), cardiac anomalies (17%) and skeletal abnormalities (7%). Importantly, the behavioral tests of the heterozygous mice showed that the mice were deficient in long-term memory (100%); in contrast, short-term memory appeared to be normal. They also showed poor locomotor activity (100%). The homozygous *CBP* mutant mice (background: 75% C57BL/6, 25% CBA or 87.5% C57BL/6, 12.5% CBA) died at E9.5 to 10.5 and showed defective neural tube closure, a lack of vascular network formation, and an inhibition of primitive hematopoiesis in the yolk sac and complete blockage of definitive hematopoiesis developing in the para-aortic splanchnopleural mesoderm region and impaired vasculo-angiogenesis (Oike, *et al.*, 1999b).

A detailed analysis of *CBP*^{+/-} mice (background: C57BL/6) generated by replacement of amino acids 340-443 with *neo* gene was also described recently (Kung *et al.*, 2000). Defects included growth retardation, craniofacial abnormalities, dramatic splenomegaly, e.g. grossly enlarged spleen. Significantly, the defects in hematopoietic differentiation and an increased incidence of hematologic malignancies with advanced age were also found in the *CBP*^{+/-} mice, but not in *p300*^{+/-} (described below) and wild-type mice. It suggests that a full complement of CBP, but not p300, is required for normal hematopoietic differentiation and that CBP appears to contain tumor suppressor activities.

Mice lacking both copies of *p300* die *in utero* between E9 to 11.5. Mice double-heterozygous for *CBP* and *p300* mutants have a comparable phenotype to either *CBP* or *p300* null mutants (Yao *et al.*, 1998). This suggests that these two proteins exert certain common embryonic survival functions and that the combined dose of CBP and p300 is critical for mouse embryonic development, although CBP and p300 are not completely physiologically redundant. The *p300* mutant mice were acquired by either an inbred cross with 129/Sv or a cross with C57BL/6 mice. The more C57BL/6 background was present, the less lethality among *p300* heterozygous animals was observed, indicating that there is a modifier gene(s) in C57BL/6 mice that suppresses the lethality observed in 129/Sv background. The embryos nullizygous for *p300* were much smaller than their littermates and exhibited severe open neural tube, as well as heart defects (an enlarged heart cavity) as observed in ~20% of the *p300*-deficient embryos at E10.5. Remarkably, there was also a significant embryonic lethality in mice lacking just one copy of *p300* (as early as at E10.5) and *CBP* (lower number of viable $CBP^{+/-}$ mice than $p300^{+/-}$ mice). A significant number of the *p300* heterozygotes displayed symmetrical exencephaly mostly in the midbrain and in the anterior part of the hindbrain region, with the rest of the neural tube fused normally. This partial penetrance of exencephaly was compatible with the restricted lethality observed among the heterozygous mutants. The heart development was perturbed in embryos lacking one or both copies of *p300*, and this failure of the cardiovascular system may, at least in part, be responsible for the observed embryonic lethality. The *p300*-deficient mice also showed that p300 is essential for normal cell proliferation *in vivo* and *in vitro* and functions as a critical cofactor of retinoic acid receptor (RAR)-, but not of CREB-mediated signaling pathway. This indicates that the physiological functions of p300 and CBP do not fully overlap, at least during embryonic development. Thus, a reduction in the dose of one of them cannot be fully compensated by a call on the other to overcome the functional deficit.

Thus, the importance of CBP during development is underscored by the occurrence of the *CBP* haplo-insufficiency disorder called RTS in humans. The heterozygous *CBP* mice (Tanaka *et al.*, 1997; Kung *et al.*, 2000) exhibit only mild RTS phenotypes in comparison to the phenotypes observed in the mouse model generated by Oike *et al.* (1999), which leads to a truncated CBP protein (amino acids 1-1084). Thus, a new mechanism by which CBP defects lead to RTS phenotypes was proposed: abnormal CBP derived from the mutant allele might interfere with wild-type CBP function in a dominant manner.

1.3.6 CBP and Rubinstein-Taybi Syndrome (RTS)

If a mutation in human *CBP* is present in all cells of the individual, Rubinstein-Taybi Syndrome (RTS) emerges. RTS is a heritable human developmental disease, characterized by multiple abnormalities, including mental and growth retardation (94~100%), associated with characteristic craniofacial appearance, including hypoplastic maxilla with narrow palate (100%), downward slanting palpebral fissures (100%) and large anterior fontanel (64%); skeletal defects (broad thumbs and broad big toes) (100%). Additional symptoms include microcephaly (84%), stiff gait, electroencephalographic abnormalities (60%), agenesis of corpus callosum and congenital heart defects (33%) (Rubinstein and Taybi, 1963; Jones *et al.*, 1988; Hennekam *et al.*, 1990). Thus, RTS is a multiple congenital malformation and mental retardation syndrome caused by a generalized dysregulation of gene expression. Magnetic resonance imaging (MRI) study of the brain of a RTS patient revealed bilateral rolandic cortical clefts and diminished white matter, which probably account for mental retardation (Sener, 1995). The typical features of the syndrome develop progressively, beginning in early infancy and reaching the fully developed phenotype

by the age of two years. The syndrome is surprisingly common, the prevalence of RTS has been estimated to be about 1 in 125,000 living newborns (Hennekam *et al.*, 1990), and it is important to note that RTS has also been estimated to account for as many as 1 in 300 institutionalized subjects with mental retardation over the age of 5 years (Padfield *et al.*, 1968; Roldan 1969). Since the recurrence risk for offspring of affected persons is estimated at 50%, an autosomal dominant inheritance pattern has been favoured. Verbal abilities are limited and intelligence is usually low. The average IQ value of the patients is below 40, and the average age and range for attainment of several developmental milestones such as standing, walking or speech development are severely delayed (Hennekam *et al.*, 1992). Behaviourally, the patients show a shortened attention span and impaired executive functions, expressed as difficulties in planning motor acts, and the execution of locomotor and oculomotor skills is disturbed (Gotts and Liemohn, 1977).

However, the knowledge about the physiological features of RTS is very limited. It is hoped that the behavioural analysis of a genetic mouse model of the disease may help to elucidate the molecular mechanisms for these phenotypes. Genetically, RTS has been shown to be associated with the long arm of chromosome 16 (16p13.3), including deletions, translocations, or point mutations. Two point mutations associated with RTS were characterized, and these two point mutations were located in the *CBP* gene (Petrij *et al.*, 1995). Two patients had a premature stop codon at amino acid positions 136 and 357, leading to truncated products missing most of the 2441 amino acid 265 kDa CBP protein. In 12% of the RTS cases, small deletions in 16p13.3 have been observed (Taine *et al.*, 1998). Moreover, *de novo* somatic chromosomal re-arrangements account for about one-fourth of these cases (Breuning *et al.*, 1993; Hennekam *et al.*, 1993). Translocations of *CBP* to other chromosomal loci can also generate aberrant fusion

proteins leading to e.g. acute myeloid leukemia (Borrow *et al.*, 1996) and other diseases (Giles *et al.*, 1998). Although some of the *CBP* mutations may generate dominant-negative isoforms, most of the evidence suggests that RTS is a haplo-insufficiency disorder.

Taken together, a genetically modified mouse model is a very useful tool to elucidate the physiological functions of CBP, and to understand the complex mechanisms underlying RTS. A molecular mechanism to explain the RTS symptoms was proposed based on the fact that CBP is a multiple transcriptional cofactor, for example, with *Ci* and *Gli-3*, which are critical for embryonic pattern formation, and with hypoxia-inducible factor 1 (HIF-1) which is responsible for neural tube closure. However, for most RTS symptoms, for example, developmental retardation and impairment of long-term memory, the molecular mechanisms still remain unclear. However, CBP, as a molecular integrator, functions as a coactivator of many other transcription factors, including CREB, which is a central factor required for long-term memory. *In vitro* experiments using a Gal4-GFP system, the ability of CBP tethered to the promoter was examined by means of fusion to the Gal4 DNA-binding domain, indicating that the activation of NMDA receptors and voltage-sensitive calcium channels leads to CREB-mediated transcription in cortical neurons via regulation of CBP (Hu *et al.*, 1999). The recruitment of CBP by CREB is a crucial step for the induction of gene transcription following Ca^{2+} flux through ligand- and/or voltage-gated ion channels in hippocampal neurons (Hardingham *et al.*, 1999). Furthermore, Marek *et al.* (2000) presented a genetic and electrophysiological analysis of *Drosophila* CBP (dCBP) function in the neuromuscular junction (NMJ), which showed that postsynaptic dCBP is necessary and may also be sufficient for the development of normal presynaptic transmitter release at the NMJ, and that dCBP can inhibit the development of presynaptic transmitter release

when overexpressed either pre- or post-synaptically. This indicates that dCBP-dependent mechanisms can act as both positive and negative regulators of presynaptic development. Although the precise relationship between CREB and CBP in the control of synaptic plasticity, learning and memory remains unclear, the mental retardation of RTS caused by mutations in *CBP* could be due to the impairment of CREB-mediated gene expression in the central nervous system. Because CBP interacts with so many different transcription factors, possibly at the same time point, it is difficult to dissect the complex components of the RTS phenotype into distinct contributions from various individual transcriptional pathways. It is hereby evident that conditional *CBP* knock-out mice in different tissues and at different time points will be a powerful model to pinpoint the mechanisms of the multiple congenital malformation and mental retardation syndrome observed in the RTS.

1.4 Gene targeting

Since the first transgenic mouse was reported in 1980, genetically engineered mice have become an invaluable biological tool for better understanding of physiological and pathological processes in many fields of biomedical research. Transgenic technology allows researchers to carry out specific genetic manipulation in all cells of a laboratory animal, and makes it possible to dissect deliberately selected gene function in a living organism and to evaluate systematically complex biological process, such as embryonic development and adult behaviors. Transgenic mice as described by Palmiter *et al.* (1982) are generated by pronuclear injection of oocytes with naked DNA. This technique allows the generation of several founders within a relatively short period of time, although, there are several disadvantages (for review, see Müller, 1999): 1) vector integration occurs randomly and the neighbouring sequence may strongly influence

expression levels of the transgene; 2) the copy number of the transgene can be highly variable; 3) the regulatory elements included in the injected DNA may be located at a great distance from the coding sequence or within introns of large genes, thus, it is not sure that the transgene does follow the expected expression pattern.

Concomitantly, gene targeting, defined as the introduction of site-specific modification into the genome by homologous recombination, has been developed and has revolutionized the field of mouse genetics. It allows the analysis of diverse aspects of gene function in embryonic development and adult physiology. In 1981, two groups (Evans and Kaufmann, 1981; Martin, 1981) acquired from mouse blastocysts totipotent embryonic stem cell (ES cell) lines, which maintain the capacity to colonize every cell lineage including germline when injected into host blastocysts under appropriate conditions (Bradley *et al.*, 1984). Concomitantly, Capecchi and his colleagues showed that homologous recombination can occur in mammalian cell lines (Folger *et al.*, 1982), paving the way to altering the mouse genome at any chosen locus by homologous recombination in ES cells. Combining both accomplishments (i.e. embryonic stem cells and homologous recombination) led to the first gene targeting experiments in mouse (Thomas and Capecchi, 1987; Doetschman *et al.*, 1987). Gene targeting of this first generation, here referred to as conventional knock-out, leads to inactivation or modification of a gene of interest in all tissues in a living organism from the onset of development throughout the whole lifespan. This technology is widely used in all fields of biological research in mouse, including embryonic development, cancer research, immunology and neuroscience (e.g. Silva *et al.*, 1992; Grant *et al.*, 1992). In the field of neurosciences, knock-out mutants have contributed greatly to the understanding of basic mechanisms of brain function involved e.g., in learning and memory (Bourtchuladze *et al.*, 1994), as well as to generate useful animal models for studying human neurological

disorders (Brusa *et al.*, 1995; Acampora *et al.*, 1996; Mansouri *et al.*, 1996; Brunet *et al.*, 1996; Timpl *et al.*, 1998). Despite the enormous progress that has been made by this technology, some intrinsic limitations are evident.

Thus, this method has been further developed with the aim of controlling the modification of a gene in a time- and tissue-dependent manner, here referred to as conditional knock-out. This is particularly useful in cases where conventional gene targeting leads to lethality in early developmental stages, thus, preventing phenotypic analysis in adults. Since a given gene often has a broad expression pattern, tissue-specific knock-out may define physiological roles of the gene in a certain tissue, without compromising functions in other tissues of the organism; knock-outs in a time dependent manner may allow us to analyze gene functions at different time points during development and in adult life. Moreover, conventional knock-out may lead to unexpected phenotypes, due to compensation mechanisms, since mammals are generally equipped with well organized regulatory systems which may overcome naturally occurring disbalancies. The disruption of a gene at early developmental stage could induce compensation mechanisms, which could then mask some physiological functions of the gene in adulthood. Such mechanisms range from simple overexpression of other related genes with similar functions, to mis-expression of non-related genes (for review see Gerlai, 2000). Keeping gene function intact throughout ontogeny, time-specific knock-outs in adulthood might avoid such compensatory responses.

Tissue- and time-specific knock-out mice can be generated by using the Cre/loxP system (for review, see Sauer, 1998). Cre is a 38 kDa site-specific DNA recombinase isolated from bacteriophage P1 (Sternberg and Hamilton, 1981). loxP (locus of crossover in P1) was originally found in the P1 genome, and is a sequence 34 bp long, consisting of two 13 bp inverted repeats flanking an 8 bp non-palindromic core

sequence that indicates the polarity of the loxP site (Fig. 1.6). Two mouse lines are required for the generation of conditional knock-out mice. One is called a “floxed” mouse line (the sequence of interest flanked by two loxP sites), and such a mouse line should not display any phenotypical changes, and is therefore considered as a wild-type mouse. These two loxP sites are introduced into 5’ and 3’ ends of essential exons of the gene of interest by homologous recombination in ES cells, the cells are then injected into blastocysts to generate finally the floxed mouse. The second mouse line that is required expressing Cre recombinase, driven by regulatory sequences of interest in a defined tissue and/or at a distinct time point.

When these two mouse lines are crossed, Cre recombinase will recognize the two loxP sites and excise or invert the sequences between the loxP sites. Cre-mediated recombination between two directly repeated loxP sites results in the excision of the sequence between them as a covalently closed circle (Fig. 1.6, 1.7). Cre-mediated recombination between pairs of loxP sites positioned in an opposite orientation results in inversion of the intervening sequence rather than excision, and this inversion will take place continuously as long as Cre recombinase is present (Fig. 1.6).

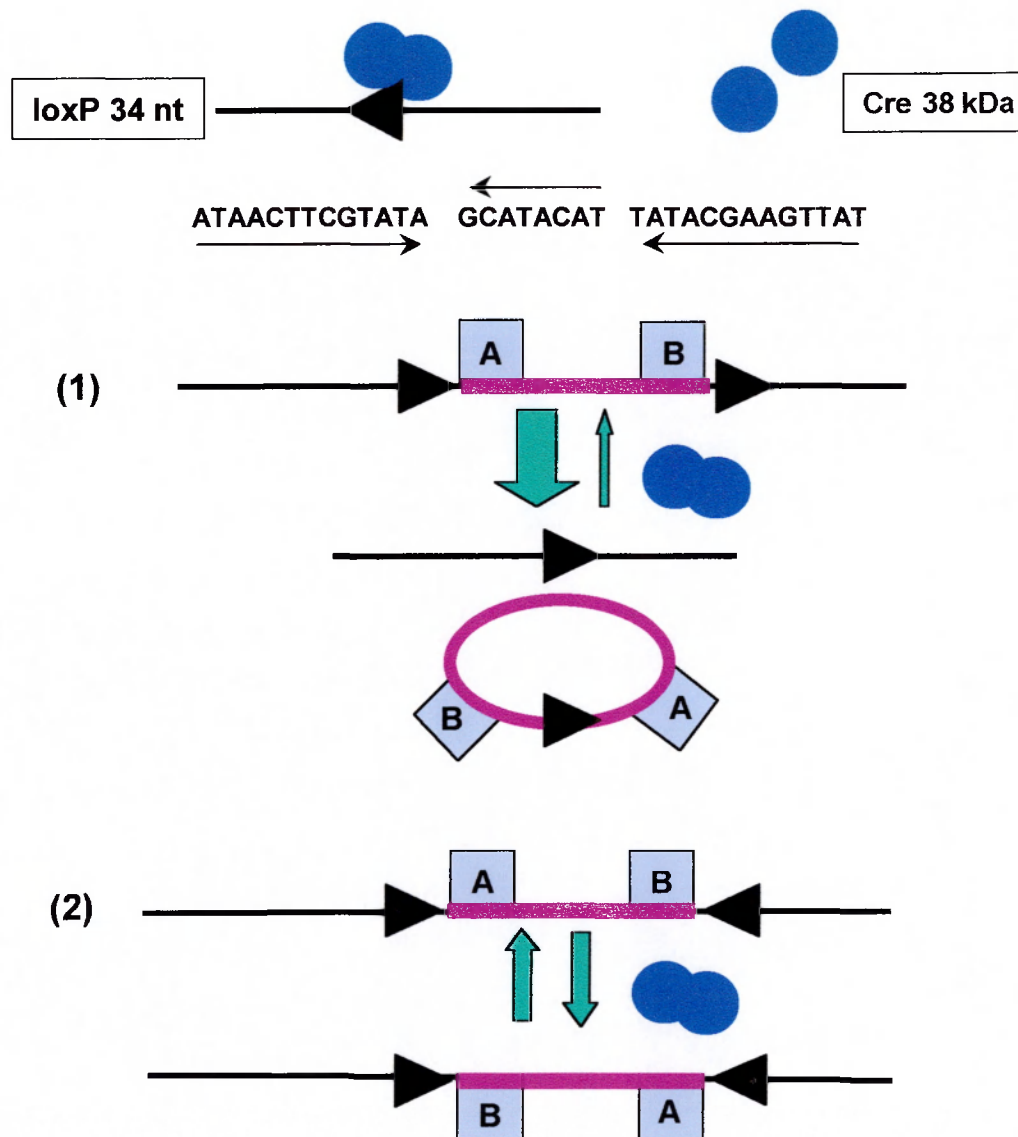


Fig. 1.6: Cre/loxP recombination system. Graphical representation of loxP site (black triangle), Cre recombinase (blue circle) and nucleotide sequence of the loxP site. Orientation of the loxP site is indicated by the orientation of the 8-nucleotide core sequence. Two molecules of Cre bind to one loxP site (Mack *et al.*, 1992). (1) If loxP sites are oriented "head-to-tail", Cre recombinase catalyzes an excision of the sequence between two loxP sites. (2) If loxP sites are oriented "head-to-head", Cre recombinase catalyzes an inversion of the sequence between two loxP sites.

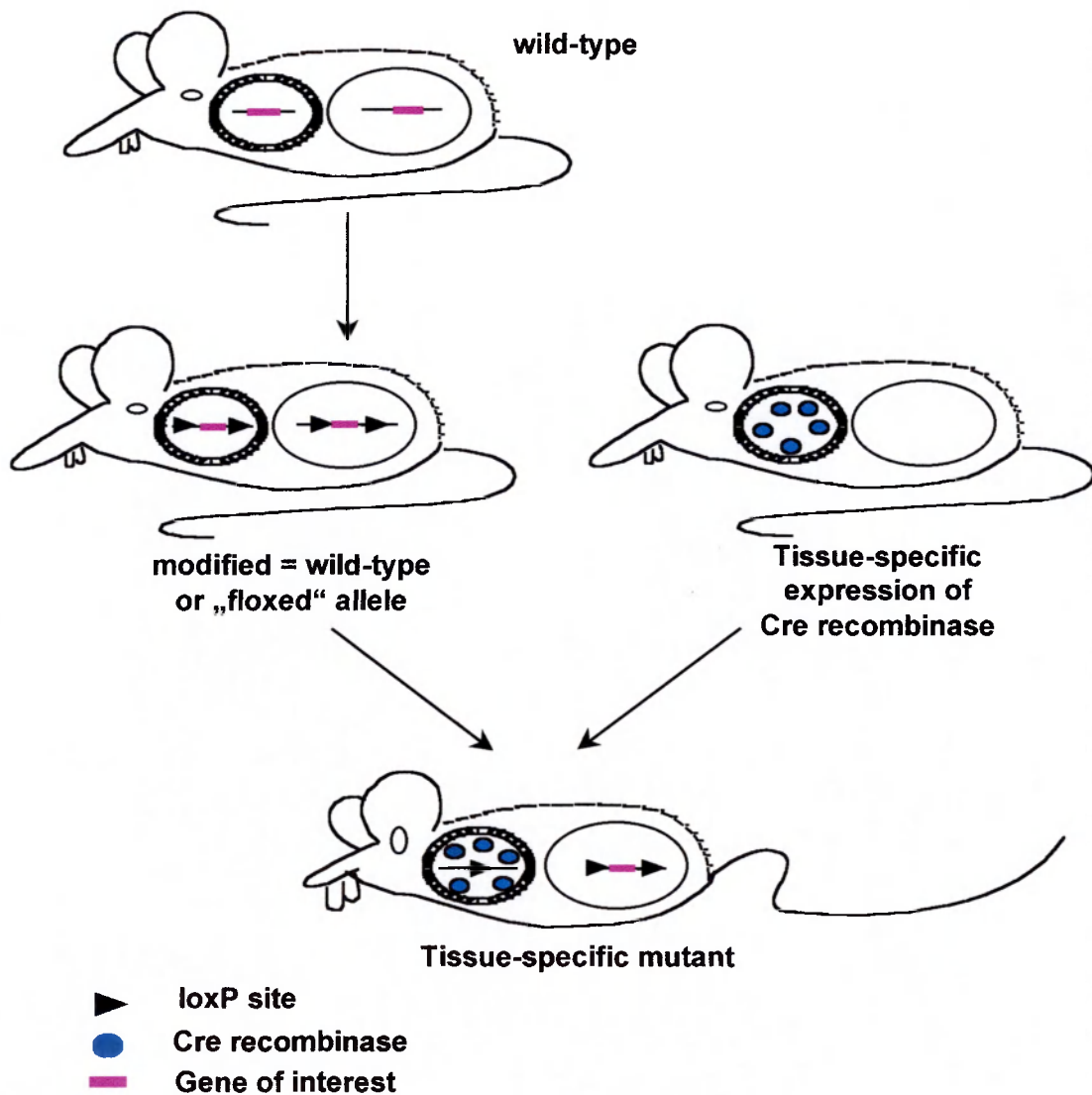


Fig. 1.7: Schematic representation of tissue-specific gene disruption using the Cre/loxP system.

Recently, Albert *et al.* (1995) further developed the Cre/loxP technique. They introduced a 5-nucleotide change into the left 13 bp element (LE) and the right 13 bp element (RE) of loxP site, generating LE mutant lox site (lox71) and RE mutant lox site (lox66), respectively. Inversion recombination between the lox71 and lox66 sites produces a wild-type loxP site and a LE+RE double mutant loxP site that is poorly recognized by Cre recombinase. This technique offers some interesting applications: 1) Integration: the frequency of site-specific integration via the mutant lox sites reached a maximum of 16%, showing high stable integration. In contrast, the wild-type loxP sites yielded very low frequencies (<0.5%) of site-specific integration events (Araki *et al.*,

1997); 2) Inversion: this technique has not yet been applied. As proposed in this thesis, a conditional CBP point mutation tyrosine to alanine at amino acid 658 (CBP^{Tyr658Ala}) can be introduced by Cre-mediated inversion with two mutant loxP sites (lox66 and lox71). With luciferase as report gene, Cre-mediated recombination has been shown to be 4 to 7-fold more efficient between lox66 and lox71 than that between double mutated loxP and wild-type loxP under the different Cre concentration used (0.1-20 µg) in tobacco protoplasts; at lower Cre concentration, the recombination between lox66 and lox71 was favoured (Albert *et al.*, 1995). The strategy of the Cre/loxP mutant system is indicated in Fig. 1.8.

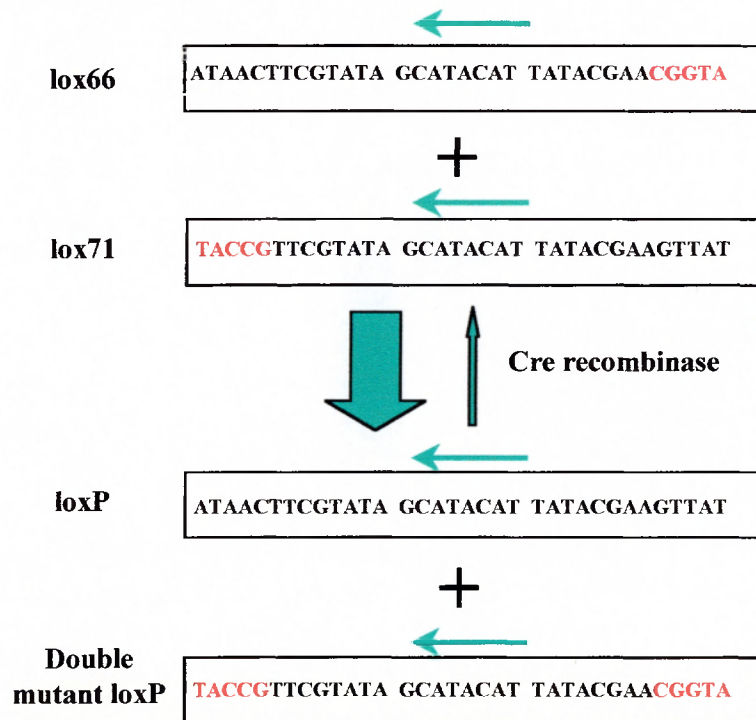


Fig. 1.8: The mutated Cre/loxP system. Nucleotide sequences of loxP and its mutants are listed, and the mutated sequences are marked in red. Recombination between lox66 and lox71 sites produces a wild-type loxP and a double mutated loxP sites. Since the double mutated loxP site exhibits reduced binding affinity for Cre recombinase, thus, Cre-mediated recombination in the mutated Cre/loxP system is preferred for the direction as indicated.

In addition to Cre/loxP, Flp/rtt system is another commonly used recombination system, which originally derived from the yeast *Saccharomyces cerevisiae* (Babineau *et al.*, 1985). Both have been shown to catalyse excisions, integrations, or translocations of DNA between their distinct recognition target sites loxP and rtt, respectively, without requirement of additional cofactors. Flp recombinase (Flipase) functions efficiently at 30°C, but less efficient than Cre recombinase at 37°C. However, Flipase still maintains recombinase activity sufficient for Flp-mediated recombination in mammalian cells. rtt (flipase recognition target), similar to loxP, is 34 bp long, consisting of two 13 bp inverted repeats flanking an 8 bp non-palindromic core sequence that indicates the polarity of its site. As for the Cre/loxP system, the type of the recombination reaction is determined by the orientation of target sites relative to each other on a segment of DNA, specifically, directly repeated rtt sites specify excision of the intervening DNA.

1.5 Aims of the thesis

Several lines of evidence demonstrate that CBP might play an important role in the transfer of learned information from short-term memory to long-term memory. (1) CBP is a major cofactor in the CREB-mediated transcriptional response, which is initiated by learning, and is required for the formation of long-term memory; (2) CBP is activated by increased intracellular concentrations of cAMP and Ca^{2+} through the kinases PKA and CaMKIV, respectively. As CaMKIV was reported to be restricted mainly to the nucleus of neurons (Means *et al.*, 1991; Matthews *et al.*, 1994), and catalytically inactive mutants of CaMKIV expressed in AtT20 cells and hippocampal neurons block Ca^{2+} -activated, CREB/CBP-mediated transcriptional response (Hardingham *et al.*, 1999; Chawla *et al.*, 1998), CBP might play a crucial role in synaptic plasticity; (3) the human

Rubinstein-Taybi Syndrome (RTS) is caused by mutations in the *CBP* gene and is characterized by mental retardation.

In order to analyse the physiological functions of CBP in neurons, I aimed to inactivate the *CBP* gene in a tissue-specific manner by using the Cre/loxP system, in the hippocampus, a brain region essential for learning and memory. Conditional knock-outs are essential, as conventional knock-outs of CBP in mice lead to embryonic lethality. In addition, since CBP is a co-factor of a variety of transcription factors and kinases, and acts as a molecular integrator of many signaling pathways. I aimed to inactivate only the CREB-binding domain (CBD) of CBP.

Expression analysis of CBP In order to investigate the functions of CBP in CNS, it is necessary to characterize its expression pattern. This is especially important in the light of the generation of tissue-specific *CBP* knock-out mice. So far, the expression pattern of mouse CBP has not been described. Therefore, I investigated the expression of CBP and its highly related protein p300, in particular, in forebrain areas essential for learning and memory. *In situ* hybridization and immunohistological experiments showed particularly high expression of both CBP and p300 in the so-called limbic system, i.e. neocortex, hippocampus, entorhinal cortex and amygdala.

Mutational analysis of CBP in vitro Transfection assays constitute a useful tool to obtain insights into possible physiological functions of CBP. In order to show whether it is possible to remove only the CREB-binding domain (CBD) of CBP and to keep other activities such as steroid hormone receptor-mediated activities, and to identify mouse mutant phenotypes that might result specifically from disruption of the CREB-CBP interaction, several mutants were generated: one that lacks most of CBD (CBP Δ CBD2-5); the second, which lacks the C-terminal domain of CBP, retaining only the first N-terminal 1100 amino acid residues, and displaying dominant-negative effects; and the

third, the point mutation Tyr658Ala (CBPAIa658), which abolishes CREB-binding activity *in vitro*. Lastly, three RTS-like mutants were generated, containing different C-terminal truncations of CBP. I then tested whether these mutants lack either cAMP-mediated or glucocorticoid-mediated transcriptional enhancement in F9 teratocarcinoma cells. Both CBPΔCBD2-5 and CBPAIa658 have a dominant-negative effect on cAMP signaling. This effect of CBPAIa658 was shown to be dose-dependent. As expected, glucocorticoid-mediated transcriptional enhancement was retained in both of the mutants CBPAIa658 and CBPΔCBD2-5. The three RTS-like mutants displayed dominant-negative effects on CBP function in the context of cAMP signalling.

Generation of mouse CBP mutants The central part of this thesis was the generation of two mouse mutants of the *CBP* gene using the Cre/loxP system:

1) Generation of a mouse model for RTS CBP^{stop523} is characterized by a mutation in exon 2 of CBD, where a new stop codon was introduced at amino acid 523 disrupting the open reading frame of the downstream sequences of CBD. It aims to mimic a CBP mutation found in human RTS patients. When this mutation is present in all cells of the animal in a heterozygous state, some RTS-like phenotypes, e.g., developmental retardation, embryonic lethality and smaller size, were observed. To dissect the complex mechanisms underlining RTS, the generation of tissue-specific heterozygous knock-outs of this allele has been initiated.

2) Generation of a CREB-binding mutation of CBP The second mutant generated is a tyrosine to alanine substitution at amino acid 658, which is reported to abolish CREB-binding activity of CBP *in vitro* (Radhakrishnan *et al.*, 1997). When crossed with Cre-expressing mice, this point mutation should be generated in a conditional tissue-specific manner. Proof of principle of this novel technique was obtained in bacteria and embryonic stem cells.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Table 2.1 Common chemicals

Chemicals	Company (Catalog number)
α -Iodoacetamid	Calbiochem, USA (407710)
2-Mercaptoethanol	Roth, Germany
2-Mercaptoethanol (for cell culture)	GIBCO, Germany (31350-010)
Acetic anhydride	Sigma, Deisenhofen, Germany (A0933)
Acrylamide (29:1)	Roth, Germany (A 515.1)
Agar, agarose	GibcoBRL, Germany
Ampicillin (sodium salt)	Sigma, Deisenhofen, Germany (K029.2)
APS (ammonium peroxodisulfat)	Roth, Germany (9592.2)
Bromophenol blue	MERCK, Germany (1.08122.0005)
Casamino acids	Sigma, Deisenhofen, Germany
Chloramphenicol	Sigma, Deisenhofen, Germany (C-0378)
Chloroform	MERCK, Germany (6340.2)
DEPC (diethylpyrocarbonate)	Roth, Germany (K028.1)
Dextran sulfate	Sigma, Deisenhofen, Germany (D-8906)
DMSO (dimethylsulfoxide)	Sigma, Deisenhofen, Germany (D-5879)
Drying pearls	Roth, Germany (0728.1)
DTT (dithiothreitol)	MERCK, Germany (1.120.130.001)
EDTA (ethylenediaminetetraacetic acid)	Sigma, Deisenhofen, Germany (E5134)

Ethanol	MERCK, Germany (1.00983.1000)
Ethidium bromide	Sigma, Deisenhofen, Germany
Ficoll 400	Sigma, Deisenhofen, Germany (F2637)
Formamide	MERCK, Germany (1.09684.2500)
Gancyclovir	Cytovene, Syntex, USA
Gelatin	BDH, England (44045)
Geneticin G418	GIBCO, Germany (11811-03)
Glutaraldehyde (25%)	Serva, Heidelberg, Germany (23115)
Guanidium isothiocyanate	Roth, Germany (0017.2)
Hydrochloric acid	Roth, Germany
Hydrogen peroxide (30%)	MERCK, Germany (1.072.100.250)
IPTG (isopropyl- β -thiogalactopyranoside)	Roth, Germany (2316.2)
Isopropanol	Roth, Germany
L-Glutamine	GIBCO, Germany (25030-024)
LIF (Leukemia Inhibitory Factor)	GIBCO, Germany (13275-029)
Maleic acid	MERCK, Germany (8.003.800.500)
Maltose	Roth, Germany (8951.1)
Methanol	Roth, Germany (4627.2)
MgSO ₄	MERCK, Germany (1.05883.0500)
N'-N-dimethyl-formamide	MERCK, Germany (D-8654)
Paraformaldehyde	MERCK, Germany (1.045005.1000)
PEG 8000	Sigma, Germany (P-5413)
Penicillin-Streptomycin	GIBCO, Germany (15140-114)
Phenol	Roth, Germany (0038.2)
Puromycin	Sigma, Germany

Retinoic acid (RA, all trans)	Sigma, Germany (R-2625)
Saponin	Sigma, Germany (S-7900)
Scintillation solution	Roth, Germany (0016.2)
Sodium acetate	MERCK, Germany
Sodium citrate	Sigma, Deisenhofen, Germany
SDS (sodium dodecyl sulfate)	Roth, Germany (2326.2)
Sodium N-lauryl sarcosine	MERCK, Germany
Spermidine	Sigma, Deisenhofen, Germany (S-0266)
TEMED	Roth, Germany (2367.3)
TEA (Triethanol amine)	Roth, Germany (6390.1)
Tris (Trihydroxymethyl-aminoethane)	Riedel-de Haen, Seelze, Germany (33742)
Trypton	GibcoBRL, Germany (50014-034)
Tween-20	Sigma, Deisenhofen, Germany (P-9416)
X-Gal	Roth, Germany (2315.2)
Xylene cyanol	MERCK, Germany (1.105900.0005)
Yeast extract	GibcoBRL, Germany (30393-029)

2.1.2 Table 2.2 Antibodies and enzymes

Antibodies/enzymes	Company (Catalog number)
Anti-CBP (A-22) sc-369	Santa Cruz Biotechnology, Inc., USA
Anti-CBP (C-1) sc-7300	Santa Cruz Biotechnology, Inc., USA
Anti-Digoxigenin-AP	Roche, Germany (1093274)
Anti-Digoxigenin-POD	Roche, Germany (851299923-07)
Anti-HA (F-7) sc-7392	Santa Cruz Biotechnology, Inc., USA
Anti-p300 (C-20) sc-585	Santa Cruz Biotechnology, Inc., USA

Cloned Pfu polymerase	Stratagene, Heidelberg, Germany
DNase I (RNase-free)	Roche, Germany (84105421-23)
MMLV reverse transcriptase	GibcoBRL, Germany (28025-013)
Mung Bean Nuclease	New England BioLabs, USA (250S)
Proteinase K	Roth, Germany (7528.2)
Restriction enzymes (incl. 10x Buffer)	New England BioLabs, USA GibcoBRL, Germany Roche, Germany
RNase A	Roche, Germany (109142)
RNasin	Promega, USA (N251B)
Shrimp alkaline phosphatase (SAP)	Roche, Germany (1758 250)
Streptavidin-AP	Roche, Germany (85777720)
T3-, T7-RNA polymerase	Roche, Germany (85121525- 22/85502020-45)
T4 DNA ligase	New England BioLabs, USA (202S)
T4 DNA polymerase	New England BioLabs, USA (203S)
T4 polynucleotide kinase	New England BioLabs, USA
Taq DNA polymerase	Roche, Germany (1647679)
Vent DNA polymerase	New England BioLabs, USA (254S)

2.1.3 Table 2.3 Nucleotides and nucleic acids

Nucleotide/Nucleic acid	Company (Catalog number)
Deoxynucleotide (dATP, dCTP, dGTP, dTTP)	Roche, Germany (1277049)
DIG RNA labeling mix	Roche, Germany (1277073)

DNA-Marker: X	Roche, Germany (1498037)
Smart Ladder	Eurogentec, Belgium (MW-1700-02)
Herring sperm DNA	Roche, Germany (85346024-39)
Ribonucleotide (rATP, rCTP, rGTP, rUTP)	Roche, Germany (85730020-50)
[α - ³² P]-dCTP	Amersham, Germany
[α - ³⁵ S]-UTP	NEN Lifescience, Boston, USA (NEG039H)
tRNA	Roche, Germany (84694421-87)

2.1.4 Table 2.4 Kits and special materials

Kits and special materials	Company (Catalog number)
Antifoam A Emulsion	Sigma, Germany (A-6457)
Autoradiography Emulsion NTB-2	Kodak, New Haven, USA
Blocking reagent	Roche, Mannheim, Germany (1096176)
BSA (Bovine serum albumin)	Sigma, Deisenhofen, Germany (8076.2)
HCG (human chorionic gonadotropin)	Intervet GmbH, Germany (27541)
LIQUID DAB Concentrated Substrate	BioGenex, Germany (HK 153-5K)
5x lysis buffer (for transfection assay)	Promega, USA (E397A)
NZ Amine (caseine hydrolysate)	Sigma, Germany (A-2427)
NZY broth	GIBCO, Germany (13635-024)
peqGOLD RNAPure™	peqlab, Erlangen, Germany
PMSG (pregnant mare serum gonadotropin)	Intervet GmbH, Germany (28509)
QIAprep Plasmid Purification Kit (Midi, Maxi and Endo-free)	QIAGEN, Hilden, Germany

QIAquick Nucleotide Removal Kit	QIAGEN, Hilden, Germany
Random primer labelling kit	GIBCO, Germany
Rapid-hyb buffer	Amersham Life Science, UK (NIF939)
RNeasy mini kit	QIAGEN, Hilden, Germany
Swine serum	Vector Laboratories Inc., USA (S-4000)
Trypsin/EDTA (1x)	0.25% trypsin, 1 mM EDTA (25300-054, GIBCO, Germany)
Trypsin inhibitor (from soy bean)	Sigma, Germany (T-9003)
TSA™ Biotin System	NEN™ Life Science Products, Inc., Boston, USA (NEL700)
Vector® Red Alkaline Phosphatase Substrate Kit I	Vector Laboratories, Burlingame, USA (SK-5100)

2.1.5 Table 2.5 Commonly used media, buffers and stock solutions

Mediums/Buffers/Stock solutions	Recipe
0.1x TE plus RNaseA	1 mM Tris-HCl pH 7.2 0.1 mM EDTA 100 µg/ml heat-inactivated RNaseA
1 M Na-phosphate buffer	257.3 g/l Na ₂ HPO ₄ ·12H ₂ O 38.5 g/l NaH ₂ PO ₄ ·H ₂ O, pH 7.2 autoclaved
10x PBS	120 mM NaCl 2.7 mM KCl 7 mM Na ₂ HPO ₄ 3 mM NaH ₂ PO ₄ , pH 7.4, autoclaved

10x TBE	1 mM Tris-Base
	0.83 mM boric acid
	20 mM EDTA, pH 8.0, autoclaved
20x SSC	3 M NaCl
	0.3 M sodium citrate, pH 7.0, autoclaved
2x freezing medium	50 % FCS
	20 % DMSO, in DMEM
5x Loading buffer	20% Ficoll 400
	0.05% bromophenol blue
	0.05% xylene cyanol
	40 mM EDTA
50x TAE	2 M Tris-Base
	1 M sodium acetate
	50 mM EDTA (pH 8.0), autoclaved
Ampicillin (stock of ampicillin Na-salt)	100 mg/ml in 75% ethanol
	Stored at -20°C
Cell lysis buffer	10 mM Tris-HCl, pH 7.5
(for extraction of DNA from ES cells)	10 mM EDTA
	10 mM NaCl
	0.5% sodium N-lauryl sarcosine
	Prior to use, add 1 mg/ml Proteinase K
Denaturing solution	0.4 M NaOH
(for blotting of DNA onto membrane)	
Denaturing solution (for phage screening)	1.5 M NaCl
	0.5 M NaOH

DEPC-H ₂ O	2 ml DEPC in 2l ddH ₂ O, shaking vigorously, leaving for 4 h at RT, then autoclave twice
Developing solution D 19	Kodak, New Haven, USA
DNase stop mix	5.5 mM EDTA 0.1 M Tris-HCl, pH 8.5
dNTP-Mix	5 mM deoxyadenosine triphosphate (dATP) 5 mM deoxycytosine triphosphate (dCTP) 5 mM deoxyguanine triphosphate (dGTP) 5 mM deoxythymidine triphosphate (dTTP)
E14 complete medium	500 ml DMEM (GIBCO, Germany) (41966-029) 75 ml FCS, heat inactivated (210471, Boehringer, Germany) 5 ml L-glutamine (200 mM) (25030-024, GIBCO, Germany) 5ml penicillin (10,000 units/ml)/ streptomycin (10,000 µg/ml) (15140-114, GIBCO, Germany) 500 µl β-mercaptoethanol (50 mM, 31350-010, GIBCO) 60 µl Leukemia Inhibitory Factor (LIF) (13275-029, GIBCO, Germany)

Fixation solution	Kodak, New Haven, USA
Heat-inactivated RNaseA	Dissolve 40 mg in 4 ml 1x TE, heat for 10 minutes at 95°C. Let slowly cool down to RT. Make aliquots and store at -20°C.
Hybridization buffer	0.5 M Na-Phosphate buffer, pH 7.2 7% SDS 10 mM EDTA, pH 8.0
HyMix (for <i>in situ</i> hybridization)	50% formamide 0.3 M NaCl 20 mM Tris-HCl, pH 8.0 5 mM EDTA 10% dextran sulfate 0.02% Ficoll 400 0.02% polyvinylpyrrolidone 0.02% BSA 0.5 mg/ml yeast transfer RNA 0.2 mg/ml carrier DNA 200 mM DTT
IPTG	100 mg/ml in H ₂ O, sterile filtered, stored at -20°C
Luria Broth (LB) Medium	1.0% trypton 0.5% yeast extract 1.0% NaCl autoclaved

Neutralizing solution (for blotting of DNA onto membrane)	0.2 M Tris-HCl, pH 7.5 1x SSC
Neutralizing solution (for phage screening)	1.5 M NaCl 0.5 M Tris-HCl, pH 7.4
NTE (for <i>in situ</i> hybridization)	500 mM NaCl 10 mM Tris-HCl, pH 8.0 5 mM EDTA, pH 8.0
NZY broth	5 g/l NaCl 2 g/l MgSO ₄ ·7H ₂ O 5 g/l yeast extract 10 g/l NZ amine (caseine hydrolysate)
NZY plate	NZY broth 1.5% Difco bacto agar, autoclaved
SM buffer	0.58% NaCl 0.2% MgSO ₄ ·7H ₂ O 50 mM Tris-HCl, pH 7.5 0.01% gelatin, autoclaved
SOC-Medium	2.0% trypton 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM MgSO ₄ 10 mM MgCl ₂ , autoclaved 20 mM glucose (sterile filtered, added after autoclaving)

Solution D (for RNA isolation)	4 M guanidium isothiocyanate
	25 mM sodium citrate pH 7.0
	0.5% sodium N-lauryl sarcosine
	in DEPC-H ₂ O
Tail lysis buffer	50 mM Tris-HCl, pH 8.0
	0.1 M EDTA
	0.1 M NaCl
	1% SDS, autoclaved
TE	10 mM Tris-HCl, pH 8.0
	1 mM EDTA, pH 8.0
TENS	0.1 M NaOH
	0.5% SDS in TE buffer
Top agar	NZY broth
	0.7% agarose, autoclaved
TSS	1x LB
	10% PEG 8000
	5% DMSO
	50 mM MgCl ₂ , adjusted to pH 6.5
X-Gal	20 mg/ml in N'-N-dimethylformamide,
	sterile filtered

2.1.6 Table 2.6 Oligonucleotide primers and probes

Sequence	Position	Products
	at cDNA level*	
Z01 F 5'- ACC ACA AGT CCA TTT GGA CAA	F 811-831	Including the strong TAD
Z02 R 5'- TGC ACC AAC AGA ACC AAT TGT	R 1363-1383	of CBP

Z03	F 5'- GCA GGG CAA CAG AAT GCC ACT T	F 1381-1402	Including the CBD of
Z04	R 5'- CGT GTC CTC CGC TTT TCT TCT A	R 1991-2012	CBP
Z05	F 5'- GCT GGA AGA AGG AGA AGA CTT	F 817-837	p300 sequence
Z06	R 5'- TCC ACC CAT CAG CCC TGT GTT	R 1358-1378	homologous to TAD
Z07	F 5'- CTA GGT ACG GGG CTT CTA GTA	F 1380-1400	p300 sequence
Z08	R 5'- GTC GGA GGA GCA CGT GAG TAA	R 1958-1978	homologous to CBD
Z09	F 5'- GAA GAA AAG CGG AGG ACA CGT	F 1993-2013	Downstream sequence of
Z10	R 5'- GCC ATG CTG TTC ATC TGC ACA	R 2247-2267	CBD
Z11	F 5'- TTC CAC CAG CCC AGT CTG TAA	F 2081-2101	Amplification of sequence
Z12	R 5'- CTG AGG CCA TGC TGT TCA TCT	R 2252-2272	from exon 6 to 7
Z13	F 5'- TGC CTT TGC CAG TGA ATC GCA	F 2120-2140	Amplification of sequence
Z14	R 5'- GAG GCA TCC GTG AAG GAG AAA	R 2288-2308	from exon 6 to 7
Z15	F 5'- TCT CAA TGC CCT AG(G TA)A TGG	F 1556-70(GTA)2111-30	Generation of mutant
	GCC CCT GCC TTT GCC A	(including <i>AvrII</i>)	CBPACBD2-5
Z16	R 5'- TCT GGG ATT CCT AGG AGC TGA	R 3345-3374	
	GGA TCT ACA	(including <i>AvrII</i>)	
Z17	F 5'- AGA ATC AGC TCT TCC AAC TTC CTT	F 1638-73; 1820-39	Generation of mutant
	GGG GGC TAC CAA (exon 3) CGT TCA		CBPACBD3
	AGC CAT CTT CCC A		
Z18	R 5'- AGA GTG CTT CTA GAG TTG GCA	R 3278-3307	
	TAA GTG CCT		
Z19	F 5'- AGA ATC AGC TCT TCC AAC TTC CT	F 1638-1660	Generation of mutant
Z20	R 5'- TTT GTA TTT T(CG C)*GA TTT TCT	R 1959-1984	CBPAIa658
	CTG CT		
Z21	F 5'- GCA GAG AAA ATC (GCG)* AAA ATA	F 1960-1988	Together with Z18 (* for
	CAA AAA GA		introduction of <i>Nru</i> I site)
Z24	F 5'- GCC GAA GCT TCT CGT GAT AAC TTC		Generation of lox66 site
	GAT TAG CAT ACA TTA TAC GAA CGG T		
Z25	R 5'- ACC GGA ATT CCC GGA CTA CCG TTC		
	GTA TAA TGT ATG CT		

Z26	F 5'- GCC GAA GCT TCT CGT GTA CCG TTC GTA TAG CAT ACA T		Generation of lox71 site
Z27	R 5'- ACC GGA ATT CCC GGA CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA CGG T		
Z22	F 5'- CCT CGA CAT AAC TTC GTA TAA TGT	F at loxP site	Screening for CBP ^{stop523}
Z28	R 5'- ATC CAA TGT CTA TCT CAG TGT GCA	R 15841-15865**	right homology arm
Z29	F 5'- GAC CAA GCT TGA TCC AGA ATG GCC GAG AAC TT	F at the translation start codon of cDNA clone, including <i>Hind</i> III site in polylinker	
Z30	R 5'- ATT AGC GGC CGC TAC TGT TTG GGC AGG TTG GGT GT	R 384-407+ <i>Not</i> I	Generation of MutRTS1
Z31	R 5'- ATT AGC GGC CGC TAC TGC TGC TGT ATC AGT TTG CGT	R 1046-1068+ <i>Not</i> I	Generation of MutRTS2
Z32	R 5'- ATT AGC GGC CGC TAT AGG GCA TTG AGA GTC CTC AT	R 1548-1570+ <i>Not</i> I	Generation of MutRTS3
Z33	F 5'- GGT CCA GCT GGC AAA CTA AAT GAT GGG CTC TCA	F <i>Pvu</i> II+ 11884-11907**	Shortening of Δ exon5
Z34	R 5'- AAC TCG AGT TGA TAT CAA AGA GCA CTA AGC AAA CAA GCT	R 4417-4440+ <i>Eco</i> R V + <i>Xho</i> I**	
Z35	F 5'- TTC TGT AGG CGG AAA GAA CCA GCT	F at Neo gene	For screening of
Z36	R 5'- AGG CAA CCA GGG TGA GGA TCA TAT	R 13484-13507**	CBP ^{Tyr658Ala} right arm
Z37	F 5'- CCT CTG AAG GAG AAA CAA GCA	F 9927-9948**	Genotyping of
Z38	R 5'- ACC ATC ATT CAT CAG TGG ACT	R 10142-10162**	CBP ^{stop523}
Z39	R 5'- CTC ATT AGT GTA AGC TGT AGT CT With Z22	R 9633-9655**	Genotyping of floxed mice without neo in CBP ^{stop523}
Z40	F 5'- ATG TAA GAA CAG CCC CAA ACA With Z38	F 9415-9435**	Check for Cre-mediated deletion in CBP ^{stop523}
Z41	F 5'- CTC TGG TAA CAT TGG AAG CCT	F 1698-1718	Check of splicing pattern
Z42	R 5'- CAT CCC TTG AGA AAC CTG CAT	R 2140-2160	of inverted Mut Δ exon5
Z43	R 5'- CTT CTA GTT CTT TTT GTA TTT TCG C With Z41	R CGC+1975-96	Check of splicing pattern of inverted Mut Δ exon5

Z44	F 5'- CAT TTA TTA GCA GAG AAA ATC GCG	F 1951-72+GCG	CGC and GCG comprise the point mutation at 658
	With Z42		
B34	F 5'-GTG GAT CGA TCC TAC CCC TTG CG		Genotyping of Flipase
B35	R 5'-GGT CCA ACT GCA GCC CAA GCT TCC		deleter mouse
B42	F 5'- GAT CGC TGC CAG GAT ATA CG		Genotyping of Cre-
B43	R 5'- CAG CGC CAT CTT CCA GCA G		deleter mouse
Z101	5'- ATG AAT ACT ATC ATT TAT TAG CAG	F 1940-1969	Sequence from CREB
	AGA AAA		Ser-133 binding region

* Position at cDNA level, referring to appendix I

** Position at genomic level, referring to appendix II

2.1.7 Table 2.7 Bacteria strains

Bacteria strains	
DH 5 α	F ⁻ /endA1, hsdR17 (r _K ⁻ m _K ⁺), supE44, thi1, recA1, gyrA (Nal ^r), relA12
LE392	F ⁻ e14 ⁺ (McrA ⁻) hsdR514 (r _K ⁻ m _K ⁺) glnV44 supF58 lacY1 or Δ (lacIZY)6 galK2 galT22 metB1 trpR55
XL-1 Blue	F ⁻ ::4 recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F ⁻ proAB lacI ^q ZAM15 Tn10 (Tet ^r)]

2.1.8 Table 2.8 Plasmids

Plasmids	
CRE-luciferase	Dr. Beat Lutz (Lutz <i>et al.</i> , 1999)
GRE-luciferase	Gift from Dr. C Behl, Max-Planck-Institute of Psychiatry, Munich, Germany
p705-Cre	Dr. F Stewart, EMBL, Heidelberg, Germany (Zhang <i>et al.</i> , 1998)

pBluescript® KS - (pBS)	Stratagene, Heidelberg, Germany (212207)
pCAGGS-FLPe	Gift from Marcus Conrad, GSF, Munich, Germany (Buchholz <i>et al.</i> , 1998)
pCEV (catalytic subunit of PKA)	Dr. Beat Lutz (Lutz <i>et al.</i> , 1999)
pCre-Pac	Gift from Ralf Kist, GSF, Munich, Germany (Taniguchi <i>et al.</i> , 1998)
pGEM-7Zf(+)	Promega, USA
pHD-CREB	Dr. Beat Lutz (Lutz <i>et al.</i> , 1999)
pRc/RSV-mCBP.HA.RK	Gift from Dr. Richard H. Goodman, Vollum Institute, Oregon Health Science University (Kwok <i>et al.</i> , 1994)
pSVaZ11-PGK-Neo	Gift from Dr. Bernhard Lüscher, University of Zürich, Switzerland
pTM-loxP	Gift from Dr. Theo Mantamadiotis, DKFZ, Heidelberg, Germany
pUSEFUL (containing <i>tk</i> gene)	Gift from Dr. Allan Bradley, Baylor College of Medicine, Houston, TX, USA

2.2 Methods

2.2.1 Basic DNA/RNA methods

2.2.1.1 DNA isolation

2.2.1.1.1 Plasmid mini-preparation

From transformation plates, one single colony was inoculated in 2 ml LB medium containing 100 µg/ml ampicillin, incubated with rotation at 37°C for overnight. Then 1 ml of culture was poured into a 1.5 ml Eppendorf tube, the rest of the culture was kept at 4°C to make later a glycerol stock. The medium was removed by centrifugation at 13,000 rpm for 10 seconds in a microfuge (Heraeus, Germany). After pouring off the LB, about 50 to 100 µl of LB was left in the tube, cells were resuspended completely by vortexing, mixed well with 300 µl of TENS solution by inversion, 150 µl of 3.0 M sodium acetate pH 5.2 was added and mixed completely by vortexing. The mixture was put on ice for 5 minutes and a centrifugation step was performed at 13,000 rpm for 8 minutes at RT. 400 µl of supernatant was transferred into a fresh tube which contained 900 µl of 100% ethanol, mixed well and put on ice for 5 minutes. The plasmid DNA was pelleted by centrifugation at 13,000 rpm for 8 minutes at RT. The DNA pellet was washed once with cold 70% ethanol, and then dried for 3 minutes in a Speed Vac® Plus (BACHOFER, Reutlingen, Germany). The pellet was resuspended in 30 µl of 0.1x TE containing 100 µg/ml heat-inactivated RNaseA. 2 µl of this preparation was mostly sufficient for a enzymatic digestion in 20 µl.

2.2.1.1.2 Large scale plasmid preparation

Plasmid DNA preparations were performed by using QIAprep Plasmid Purification Kits (Midi or Maxi kits for preparation of up to 100 µg or 500 µg of plasmid DNA, respectively; Endo-Free Maxi kit for preparation of up to 500 µg of endotoxin-free plasmid DNA) (QIAGEN, Hilden, Germany). The concentration of DNA was measured at OD_{260 nm} with Spectro photometer Du®640 (BECKMAN, USA).

2.2.1.1.3 Preparation of genomic DNA from embryonic stem (ES) cells

ES cells were cultured in gelatinized 96-well plates until cells grew to confluency or the medium changed to a yellow colour. The cells were washed twice with PBS. 50 µl of lysis buffer/well was added and plates were incubated at 50°C overnight in a sealed humidified box. The plates were spun briefly to save the condensation water present in the lid. 100 µl of precipitation solution (150 µl of 5 M NaCl plus 10 ml of cold 100% ethanol) was added into each well. The plates were shaken at RT for 30 minutes. After centrifugation at 2,500 rpm for 2 minutes, the supernatant was carefully discarded by inverting the plates onto paper towels. The pellets were washed three times with 70% ethanol. Genomic DNA was stored at -20°C in 70% ethanol until use, or air dried for a few minutes and then used immediately for enzymatic digestion.

2.2.1.1.4 Preparation of genomic DNA from mouse tails

About 1 cm of tail in length was cut and incubated in 700 µl of tail lysis buffer containing 40 µl of freshly added 10 mg/ml Proteinase K overnight at 56°C in a shaking Eppendorf thermomixer (Eppendorf, Germany). Tail lysate was vortexed vigorously for 5 minutes, 300 µl of 7 M NaCl solution was added, and lysate was shaken for another 5 minutes. After centrifugation at 13,000 rpm for 10 minutes at RT, 750 µl of the upper phase was immediately transferred into a new tube, mixed well with 500 µl isopropanol by inversion and then spun at 13,000 rpm for 5 minutes at RT. Supernatant was discarded carefully using a pipette. Pellet was washed once with 1 ml of 70% ethanol, air dried for about 30 minutes and dissolved into 200 µl TE buffer by incubation at 37°C for 10 minutes and gently vortexing. DNA was stored at -20°C until use.

2.2.1.2 RNA isolation

RNA isolation from cultured cells. Cells were rinsed once with PBS. 4 ml Solution D was added to a 150 mm-dish or 1.6 ml to a 100 mm-dish. The lysate mixture was transferred into a Greiner centrifuge tube.

RNA isolation from animal tissues. After dissection, the tissue was homogenized. Solution D (1.5 ml/100 mg tissue) was pipetted into the tube containing the sample and grinded immediately using a Polytron. Prior to use, 7.2 μ l of 2-mercaptoethanol/ml (final concentration 100 mM) and 12 μ l Antifoam A Emulsion/ml were added to Solution D.

Extraction. 100 μ l acidic NaOAc (pH 4.5)/ml lysate was added and shaken well. Then, 1 ml phenol was added to the mixture, which was shaken well by inversion. Afterwards, 200 μ l of chloroform/isoamylalcohol (24:1) was added, and shaken vigorously for 10 seconds. The mixture was chilled on ice for 15 minutes. The top (aqueous) phase was transferred into a new tube after centrifugation at 13,000 rpm for 15 minutes at 4°C. An equal volume of isopropanol was added, mixed well by inversion, and then incubated at -20°C for at least 2 hours. The pellet was formed by centrifugation at 13,000 rpm for 15 minutes at 4°C, then resuspended in 500 μ l Solution D. Another 50 μ l of acidic NaOAc and 400 μ l of phenol was added and mixed well by vortexing 10 seconds. 400 μ l of chloroform was added and mixed by vortexing. The mixture was put on ice for 15 min and centrifuged at 13,000 rpm for 15 minutes at 4°C. The top phase was transferred into a new tube, mixed well with 550 μ l of isopropanol, incubated at -20°C for at least 2 hours. The RNA was pelleted by centrifugation at 13,000 rpm for 15 minutes at 4°C, then washed once with 1 ml ice-cold 70% ethanol and air-dried. The RNA pellet was resuspended in 100 μ l of DEPC-H₂O, and stored at -80°C.

RNA isolation from ES cell culture with peqGOLD RNAPure reagent. ES cells cultured on a 10 cm-gelatinized plate was rinsed twice with PBS, then, 1.8 ml of peqGOLD RNAPure solution was added. The lysate was scraped, collected into a 2 ml tube, then mixed with 0.4 ml chloroform by inverting 15 seconds and finally incubated at RT for 5 minutes. After centrifugation at 12,000 rpm for 15 minutes at 4°C, 1 ml of supernatant was transferred carefully into a fresh tube and mixed with an equal volume of isopropanol. The mixture was incubated for 10 minutes at RT. The RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. The pellet was washed twice with 1 ml 70% ethanol, air dried, resuspended in 40 µl of DEPC-H₂O, and then stored at -20°C.

2.2.1.3 Agarose gel electrophoresis

0.8-2.0% agarose gels containing 0.5 µg/ml ethidium bromide were prepared to check and/or isolate DNA fragments. DNA containing 1x loading buffer was separated for 30 minutes to overnight at about 5 V/cm in 1x TAE buffer. Gels were photographed on UV transilluminator.

2.2.1.4 PCR reaction

Table 2.9 Standard 50 µl-PCR reaction

H ₂ O	36 µl
dNTPs (5 mM each)	2 µl
10x Taq polymerase buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl ₂)	5 µl
Forward primer, 10 pmol/µl	2.5 µl

Reverse primer, 10 pmol/μl	2.5 μl
DNA template (include also a control without DNA!)	1 μl
Taq polymerase (0.5 unit/μl)	1 μl

Conditions:	95°C, 3 min	1x
	95°C, 1 min	30 to 35 times
	50-60°C, 1 min	
	72°C, 1 min	
	72°C, 5 min	1x
(for Stratagene Robocycler 96)		

2.2.1.5 RT-PCR

DNase treatment of RNA

Table 2.10 DNase mix (6 μl total)

3 μl	10x DNase buffer (200 mM Tris-HCl pH 7.2, 50 mM MgCl ₂ , 10 mM DTT)
0.25 μl	RNase-free DNase I
0.25 μl	RNasin
2.5 μl	H ₂ O

Table 2.11 DNase reaction (30 μl)

6μl	DNase mix
24 μl	RNA (2-10 μg total)

After 30 minutes incubation at 37°C, 33 µl of DNase-stop mix was added. RNA mixture was extracted with 70 µl of phenol/chloroform (1:1). The supernatant was transferred into a new tube and precipitated by adding of 1/10 volume of 3 M NaOAc (pH 5.2) and 2.5 volumes of 100% ethanol, incubating at -20°C for at least 2 hours. After centrifugation at 13,000 rpm for 15 minutes at 4°C, RNA pellet was washed with 100 µl cold (-20°C) 70% ethanol. Pellet was dried at RT for 10-20 minutes and dissolved into 10 µl DEPC-H₂O.

After DNase treatment, purification of RNA was also performed with RNeasy mini kit.

Table 2.12 RT reaction (10 µl)

2 µl	5x RT-buffer
1 µl	d(N) ₆ (100 pmol/µl)
1 µl	dNTP mix (5 mM each)
1 µl	DTT (0.1 M)
0.3 µl	RNasin
1 µl	BSA (1 mg/ml)
1 µl	MMLV reverse transcriptase (or H ₂ O for control)
3 µl	RNA (150-1000 ng) (denatured for 2 minutes at 70°C and chilled on ice, quick spin in centrifuge, then pipette above reagents as a pre-mix into this tube)

The reaction was carried out at 37°C for 30 minutes and then at 55°C for another 30 minutes. The reverse transcriptase was inactivated by heating for 4 minutes at 95°C.

PCR. 1-2 µl of RT reaction was taken for a 50 µl-PCR reaction.

2.2.1.6 Restriction enzyme digestion**Table 2.13 Standard digestion**

10x incubation buffer	2 μ l
10x BSA	2 μ l or not (depending on enzyme)
DNA (1 μ g/ μ l in 0.1x TE)	1 μ l
restriction enzyme	1 unit/ μ g DNA
H ₂ O	Added to 20 μ l

Table 2.14 Genomic DNA digestion

10x incubation buffer	3 μ l
10x BSA	3 μ l or not (depending on enzyme)
DNA (from ES cells prepared from 1 well of a 96-well plate or 15 μ g tail DNA)	x μ l
RNase (10 mg/ml)	0.3 μ l
Spermidine (0.1 M)	1.2 μ l
restriction enzyme	100 units
H ₂ O	Added to 30 μ l

Digestion mixture was incubated at 37°C or special temperature as product's description for a proper time. Genomic DNA was generally digested at 37°C or special temperature at least overnight.

2.2.1.7 Blunting of DNA**Table 2.15 Fill-in of sticky ends with T4 DNA polymerase (100 μ l)**

DNA digestion (ca. 2-5 µg total)	30 µl
10x T4 DNA polymerase buffer	10 µl
BSA (10 mg/ml)	5 µl
dNTP mix (5 mM, each)	2 µl
H ₂ O	51.5 µl
T4 DNA polymerase (3 units/µl)	1.5 µl

Mixture was incubated for 20 minutes at 11°C. Enzyme was inactivated by adding 2 µl of 0.5 M EDTA. DNA was purified by QIAquick PCR Purification kit.

Table 2.16 Removal of single-stranded extensions with Mung Bean Nuclease (50 µl)

DNA digestion (1-2 µg total)	20 µl
10x Mung Bean Nuclease buffer	5 µl
H ₂ O	24.8 µl
Mung Bean Nuclease (10 units/µl)	0.2 µl

Mixture was incubated at 30 °C for 30 minutes. DNA was purified by QIAquick PCR Purification kit.

2.2.1.8 Table 2.17 Phosphatase treatment (20 µl)

DNA digest (about 100 ng)	3 µl
10x phosphatase buffer	2 µl
H ₂ O	13 µl
Shrimp alkaline phosphatase (SAP) (1 unit/µl)	2 µl

Mixture was incubated 1 hour at 37°C for blunt-ends; and 10 minute at 37°C for sticky ends. SAP was inactivated for 15 minutes at 65°C. The reaction mix can be used for ligation directly, 20-30 ng of dephosphorylated vector is good for one ligation.

2.2.1.9 Ligation (20 µl reaction)

20 ng of vector was generally taken for a 20 µl standard ligation reaction. The amount of insert principally depends on the size ratio of vector to insert. Mostly, a molar ratio of between 3 and 6 (insert to vector) was taken.

$$\text{Insert (ng)} = 20 \text{ ng} \times \text{insert (bp)} / \text{vector (bp)} \times \text{ratio}$$

Table 2.18

Vector	20 ng
Insert	3-6 fold molar excess
10x ligase buffer (supplied with ligase)	2 µl
T4 ligase	1 µl
H ₂ O	Added to 20 µl

Mixture was incubated at 16°C for overnight, or at RT for 2 hours.

2.2.1.10 Preparation of competent bacteria

A single colony of XL1 blue or DH5α was inoculated in 1 ml LB medium and incubated at 37°C overnight. 100 ml of pre-warmed LB medium was inoculated with the overnight culture and incubated with shaking at 37°C to OD_{600 nm} = 0.3 - 0.4 (about 2 hours). After centrifugation at 3,000 rpm for 10 minutes at 4°C, supernatant was

carefully discarded, and cell pellet was gently resuspended on ice with 10 ml of ice-cold TSS solution. On ice, 250 μ l and 500 μ l aliquots were made in Eppendorf tubes, and immersed into a dry-ice/ethanol bath for a few minutes and then stored at -140°C .

2.2.1.11 Transformation

10 μ l of the ligation reaction was mixed gently with 200 μ l competent bacteria that were thawed on ice for about 15 minutes. The mixture was incubated for 30 min on ice, heat-shocked at 42°C for 90 seconds, then chilled on ice for 2 minutes. 800 μ l SOC was added, and incubated with shaking for another 30 min at 37°C . 100 μ l and 400 μ l of the reaction mix were plated onto LB plates with proper selection antibiotic(s). Plates were air-dried and incubated in an inverted position overnight at 37°C .

2.2.2 Southern blot

Blot. DNA digestions were run on a 0.8% agarose gel (containing 0.5 $\mu\text{g/ml}$ ethidium bromide) in 1x TAE buffer. After electrophoresis, gels were photographed on a UV transilluminator. Pre-treatment of gels was performed by the following steps: shaking 6 minutes in 0.25 N HCl; brief rinsing of gels with distilled water; shaking 30 minutes in denaturation buffer and then in neutralization buffer. Appropriate size of Hybond NX membrane (Amersham, USA) was immersed briefly in H_2O and 15 minutes in 10x SSC buffer, then used for transferring DNA. Capillary blot was set up according to the protocol described in “Molecular Cloning” (Sambrook *et al.*, 1989) in 10x SSC buffer for about 18 hours. After blotting, the membranes were treated 1 minute in 0.4 M NaOH and 1 minute in 0.2 M Tris-HCl/1x SSC, were then air-dried and crosslinked under the condition of “autocross link” with a Stratalinker (Stratagene, Heidelberg, Germany).

Hybridization. Rapid-hyb buffer (Amersham) and hybridization bottles were pre-warmed at 65°C. The blotted membranes were pre-wetted firstly with H₂O, then with 2x SSC, and placed onto nylon mesh. The membrane/mesh was rolled and put into hybridization bottle, then un-rolled. For the prehybridization, 10 ml pre-warmed Rapid-hyb buffer was required, for at least half a hour at 65°C. Afterwards, heat denatured (95°C, 5 minutes) ³²P-labelled probe was added into hybridization bottle directly. Hybridization was carried out at 65°C for 2 hours.

Washes. After hybridization, membranes were subjected to washing steps: rinsing briefly twice in 50 ml of 2x SSC at RT in the bottles; washing once at 65°C with shaking for 15 minutes in 500 ml of 2x SSC/0.1% SDS and 0.2x SSC/0.1% SDS, respectively; rinsing briefly once more in 2x SSC. The membranes were kept wet and sealed in plastic bags immediately, then exposed at -80°C for a few hours to a few days to autoradiographic film with an intensifying screen.

2.2.3 *In situ* hybridization

Preparation of riboprobes. After digestion, the linearized plasmid was extracted with equal volume of phenol/chloroform (1:1). After centrifugation at 13,000 rpm for 3 minutes at RT, the supernatant was transferred into a fresh tube and added with 1/10 volume of 3 M NaOAc and 2.5 volumes of 100% ethanol to precipitate the DNA. The mixture was incubated at -20°C for at least two hours and then centrifuged at 13,000 rpm for 15 minutes at 4°C, the DNA pellet was washed once with cold 70% ethanol, and air-dried for a few minutes, dissolved in 10 µl DEPC-treated H₂O. The concentration of probes was measured and then adjusted to 1.0 µg/µl with DEPC-treated H₂O.

Table 2.19 Labelling of riboprobes with digoxigenin (50 µl)

31.5 µl	DEPC-treated H ₂ O
5.0 µl	10x transcription buffer
5.0 µl	DIG RNA labelling mix
1.5 µl	linearized plasmid DNA (1.0 µg/µl)
2.0 µl	RNasin (40 units/µl)
5.0 µl	T3 or T7 RNA polymerase

The mixture was prepared at RT and was incubated at 37°C for 3 hours. Integrity of RNA was checked by running of 0.5 µl of above reaction mix on 1.0% agarose gel. The remaining 49.5 µl of the reaction was treated with 2 µl DNase, I (RNase-free) for 15 minutes at 37°C.

Purification of riboprobes. The DNase-digested reaction mix was mixed well with 5 µl of 5 M NH₄OAc and 50 µl of isopropanol, incubated 30 minutes at –20°C. After centrifugation at 12,000 rpm for 15 minutes at 4°C, the pellet was washed once with cold 70% ethanol, and air-dried for 5-10 minutes. RNA was then dissolved in 20 µl DEPC-H₂O very well by vortexing vigorously.

Quantitation of DIG-labelled riboprobes. It was achieved by using of DIG Quantitation and DIG Control Teststrips (Roche, Germany).

Pre-treatment. Cryosections (20 µm) were cut coronally from unfixed mouse brain. Pre-treatment was performed as follow: fixed for 20 minutes with 4% paraformaldehyde (PFA) in PBS; rinsed twice with 1x PBS for 5 minutes; quenched for 15 minutes with 1% H₂O₂ in 100% methanol; washed twice with 1x PBS; quenched for 8 minutes with 0.2 M HCl; washed twice with 1x PBS; digested with 20 µg/ml proteinase K (0.4

units/ml) in 50 mM Tris-HCl, 5 mM EDTA (pH 8.0) for 10 minutes; rinsed with 1x PBS for 5 minutes; fixed with 4% PFA for 20 minutes; acetylated with acetic anhydride in 0.1 M triethanol amine/HCl (pH 8.0) (by adding of 600 μ l acetic anhydride to the solution as it is being stirred in a hood, 3 minutes later, another 600 μ l acetic anhydride was added. Total acetylation treatment time was up to 10 minutes); rinsed with 1x PBS for 5 minutes; washed with 0.9% NaCl for 5 minutes; dehydrated with 30% and 50% ethanol for 20 seconds each; transferred into 70% ethanol for 1 minute; passed through 1x 80%, 1x 95%, and 2x 100% ethanol for 20 seconds each and air dried.

Hybridization. Before adding probes to the complete hybridization mix, riboprobes were placed for 2 minutes into boiling water to be denatured, then spun quickly to remove condensation water from the cap of the tube. The probes were added to the complete pre-warmed hybridization mix (HyMix/10 mM DTT/ 400 U/ml RNasin) to the final concentration of 200 ng/ml and left at 54°C in heating block (Thermomixer, Eppendorf, Germany), while it is being added onto the slides. 100 μ l of hybridization mix containing riboprobe was placed onto each slide that was covered with coverslip. This was done carefully to avoid the formation of bubbles over and around the sections. Slides were then placed into a humidified chamber containing 50% formamide/5x SSC and incubated overnight at 54°C.

Post-hybridization. Coverslips were removed and slides were firstly washed at 62°C as follow: in 5x SSC/0.05% Tween-20 for 20 minutes; 50% formamide, 2x SSC/0.05% Tween-20 for 30 minutes; 50% formamide, 1x SSC/0.05% Tween-20 for 30 minutes and 0.1x SSC/0.05% Tween-20 for 30 minutes. Then slides were washed at 30°C with NTE/0.05% Tween-20 and twice with NTE/0.05% Tween-20.

Antibody reaction. Slides were blocked 1 hour with 4% heat-inactivated sheep serum in TNT (0.05% Tween-20 in 100 mM Tris/150mM NaCl, pH 7.6), washed 3 times with

TNT, and incubated for 30 minutes with NEN-TNB blocking buffer (600-700 μ l/slide) and for 1.5 hour with anti-Dig(Fab)-POD, 1:1200 diluted in TNB (400-500 μ l/slide). Afterwards, slides were washed 3 times with TNT, incubated for 12 minutes with tyramine-biotin (TB) (TSA Individual Reagent Packs) diluted 1:50 with NEN reaction buffer (150-200 μ l/slide). Slides were washed with wash buffer (100 mM maleate/150mM NaCl/0.05% Tween-20, pH 7.5), incubated for 1 hour with streptavidine-POD antibody, 1:750 diluted with 1% Roche's blocking reagent in wash buffer (400-500 μ l/slide), washed again 3 times with wash buffer followed by two washes with TNT.

Evaluation. Reaction was visualized by incubation with BioGenex-DAB substrate at RT for up to 40 minutes and stopped by dipping in H₂O. Counterstain was performed as follow: staining 10 seconds in toluidin blue (0.1% in distilled H₂O); twice rinsing 30 seconds in H₂O; destaining 45 seconds in 70% ethanol containing 1-2 drops of 100% acetic acid and rinsing again 45 seconds in 100% ethanol. Lastly, the slides were air dried at RT and covered with HistoFluid and coverslipped.

2.2.4 Immunostaining

8 μ m thick paraffin sections were made from mouse brains. Pre-treatment was performed in cuvettes as follow: sections were deparaffinized in xylene twice for 10 minutes; washed twice with 100% ethanol for 5 minutes, once with 95% ethanol for 2 minutes and once with 70% ethanol for 5 minutes, then rinsed twice in H₂O for 2 minutes. After incubation for 15 min in 0.3% H₂O₂ in H₂O (freshly prepared) at RT, sections were washed 3 times in PBS for 2 minutes at RT, then incubated for 30 minutes in 5 μ g/ml saponin in H₂O at RT. Afterward, sections were washed 3 times in PBS for 2 minutes at RT. Slides were removed from the cuvette, drained quickly and encircled

with Pap Pen. Then, slides were placed horizontally and blocked for 20 minutes in 1.5% swine serum/PBS in humidified chamber, washed once with PBS, incubated with 100-200 μ l primary antibody appropriately diluted in 1% BSA/PBS overnight at 4°C or for 60 min at room temperature in humidified chamber. Slides were then rinsed quickly with PBS by using Pasteur pipette and wash 3 times in PBS in cuvette. Biotinylated secondary antibody was added onto the slides placed horizontally and incubated for 30 minutes at RT in humidified chamber. Slides were placed horizontally and washed 3 times with PBS for 5 minutes, then incubated with Streptavidin-Peroxidase Complex (pre-incubated for at least 30 min on ice) for 30 min at RT. Slides were again washed 3 times with PBS for 5 minutes and rinsed once with TBS (10 mM Tris-HCl, pH 7.8, 150 mM NaCl) containing 0.5% Tween-20. Diaminobenzidine (DAB)-stain was done as follow: two DAB tablets and two H₂O₂ tablets (Sigma fast DAB tablets, D-4168) were dissolved in 5 ml TBS, and vortexed vigorously for 1 minute. DAB solution was added immediately onto sections and staining was controlled through microscope. Generally, staining time was 5-15 minutes. Reaction was stopped by removal of DAB solution and transferring slides into cuvette containing H₂O. Counterstain was done with 0.1% toluidin blue (referring to Chapter 2.2.3, *in situ* hybridization). Slides were dehydrated in an ethanol series (70%, 95%, 100%) for 30 seconds for each step, washed once in xylene for 2 minutes and air dried, lastly covered with Histofluid and coverslipped.

2.2.5 Transfection

Wild-type or mutant CBP together with CRE-luciferase or GRE-luciferase reporter construct were co-transfected into F9 teratocarcinoma cells with EffecteneTM Transfection Reagent Kit (QIAGEN, Germany) to measure the luciferase activity induced by PKA or dexamethasone, respectively. 1.5×10^5 F9 cells/well were plated in

6-well tissue culture testplate (TPP, Switzerland) and treated for three days with 5 μ M retinoic acid (RA). For each well, 3.0 μ g total amount of plasmid DNA were transfected into RA pre-treated F9 cells, including 0.25 μ g pCREB, 0.25 μ g pCEV, 1.0 μ g CRE-luciferase, 1.0 μ g WT or mutant CBP, and empty vector pRc/RSV to 3.0 μ g (pRc/RSV was obtained by *Hind* III and *Not* I double digestion of pRc/RSV-mCBP.HA, isolation of the 5.2 kb vector backbone, fill-in of both sticky ends and self-ligation). 16-18 hours later, cells were fed with fresh medium containing 5 μ M RA. 2 days after transfection, the cells/well were lysed with 150 μ l 1x lysis buffer, harvested into a Eppendorf tube. After centrifugation at 13,000 rpm for 10 minutes at 4°C, 50 μ l of the supernatant was transferred into a 96-well plate for the measurement of luciferase activity in Wallac Victor_{TM}² 1420 multilabel counter (Wallac distribution GmbH, Germany).

2.2.6 Isolation of genomic *CBP* DNA

Genomic DNA fragments spanning CREB-binding domain of *CBP* were isolated from λ phage genomic DNA libraries prepared from E14 and D3 ES cells. The libraries were generated by insertion of the *Sau*3A I partially digested fragments into λ DASH II phage vector digested with *Bam*H I, provided by Dr. Klaus Kaestner, Philadelphia, USA.

2.2.6.1 Preparation of host bacteria and phage

A single colony of the host *Escherichia coli* bacterial strain LE392 was inoculated into 50 ml of LB medium supplemented with 0.2% maltose and 10 mM MgSO₄. The bacteria were then incubated at 30°C with shaking for overnight. After centrifugation of the culture at 2,000 rpm for 10 minutes at RT, the bacterial pellet was resuspended

gently in 30 ml of 10 mM MgSO_4 , then the concentration of the host bacteria was measured with Spectro photometer Du®640 (BECKMAN, USA) and adjusted to $\text{OD}_{600\text{nm}} = 0.5$ with 10 mM MgSO_4 . Definite amount of E14 or D3 phages were used for screening. 100,000 plaque forming units (pfu) was taken for the primary screening of each λ phage genomic library, and 10 times and 100 times lower numbers of phages for the secondary and tertiary screenings, respectively.

2.2.6.2 Absorption of phage into host bacteria

For primary screening, 22 x 22 cm^2 (or 10 cm diameter dishes for the secondary and tertiary screenings) NZY agar plates were prepared and pre-warmed at 42°C for at least 2 hours. The mixture of 1.8 ml LE392 ($\text{OD}_{600\text{nm}} = 0.5$) and 0.9 ml phage (appropriate amount of phages) was incubated at 37°C for 15 minutes, then 25 ml of top agar which pre-warmed at 48°C was added, mixed well and poured immediately onto each pre-warmed plate. The plates were cooled down at RT for 15 minutes, then incubated at 37°C overnight.

2.2.6.3 Blotting of phage onto membranes

When phage plaques appeared to be visible in the bacterial lawn, the plates were transferred into 4°C for hours. Two appropriately sized Hybond NX membranes (Amersham pharmacia Biotech, UK) (a little smaller than 22 x 22 cm^2) were prepared for each plate. The first one was carefully placed onto the plate, and left soaking for 2 minutes. Meantime, for orientation of the membrane and corresponding plate, eight asymmetrical holes were punched through the membrane into the NZY agar plate with a syringe needle containing black ink. After removal of first membrane, the second one was placed onto the plate for another soaking of 5 minutes. Both membranes blotted

with phage plaques were placed onto series of sheets of 3 MM filter paper (Whatman, USA) saturated by denaturation solution and neutralization solution, respectively, then committed to denaturation for 5 minutes and neutralization twice for 3 minutes each. The membranes were then submerged in 2x SSC and washed vigorously to get rid of proteinous debris. Afterwards, membranes were air-dried and UV crosslinked under the condition of “autocross link” with a Stratalinker (Stratagene, Heidelberg, Germany).

2.2.6.4 Hybridization

Hybridization buffer (referring to Tab. 2.5) and hybridization bottles were pre-warmed at 65°C. The blotted membranes were pre-wetted firstly with H₂O, then in 2x SSC, and placed onto nylon meshes. The membrane/mesh stack was rolled and put into the hybridization bottle, then un-rolled. 20 ml of pre-warmed hybridization buffer was required for the pre-hybridization at 65°C for at least one hour. Afterwards, hybridization buffer was substituted with 10 ml of fresh hybridization buffer and heat denatured (95°C, 5 minutes) ³²P-labelled probe. The probes were acquired by double digestion of the plasmid pBS-CBP.TAD and pBS-CBP.CBD (see Chapter 3; Results) with *Hind*III and *Eco*RI, then purified through the QIAquick Gel Extraction Kit. Quantitation of the probes was carried out by comparison of the extracted band with the Smart Marker on the agarose gel. 50 ng of each probe was labelled with α ³²P-dCTP for 2 hours at RT with Random primer DNA labelling system. Purification of probe was performed by MicroSpinTM S-300HR column (Amersham Pharmacia Biotech, UK). The probes were heat denatured at 95°C for 5 minutes, then immediately cooled down on ice before use. Hybridization was carried out at 65 °C for 2 hours.

After hybridization, hybridization mix (including probe) was frozen at –20°C, which was re-used up to 3 times (only for phage re-screening). The membranes were subjected

to washing steps: rinsing briefly in 50 ml of 2x SSC/0.1% SDS at RT in the bottle; 50 ml of 2x SSC/0.1% SDS at 65°C with rotating for 10 minutes in the bottle; twice in 500 ml of 0.1x SSC/0.1% SDS at 65 °C with shaking for 10 minutes; rinsing briefly once more in 2x SSC/0.1% SDS. The membranes were kept wet and sealed in plastic bags immediately, then exposed to film at –80°C with intensifying screen for a few hours to a few days for autoradiography.

2.2.6.5 Screening for genomic *CBP* DNA from phage libraries

After hybridization with CBP probe TAD and CBD, the phage plaques that showed signals on both membranes from the same plate were considered as positive. The surrounding areas (about 0.5 cm²) of the positive plaques were picked and transferred into Eppendorf tubes, each containing 1 ml of SM solution. The agar containing phages was split into pieces with tips, and incubated at RT for 3 hours. After brief centrifugation, 2 drops of chloroform was added into the phage supernatant for long-term storage of phages at 4°C.

The further screenings were carried out as the primary screening, but with the differences that more diluted phage titers were plated out, and smaller surrounding areas of positive plaques were picked from plates. Generally, four rounds of screening were required for isolation of the phages, which was considered when all plaques on one plate were positive after hybridization.

2.2.6.6 Large scale preparation of phage DNA

To prepare a large phage stock, 5 ml of SM buffer was added into a confluent phage plate (10 cm diameter), incubated at RT with shaking for 4 hours. Phage eluent was collected as completely as possible, then mixed with 0.1 ml chloroform, stored at 4°C.

After titration, 3×10^7 pfu of each positive phage in 0.5 ml SM buffer were prepared and mixed with 1×10^9 host bacteria LE392 in 0.5 ml 10 mM MgSO_4 and 0.5 ml $\text{MgCl}_2/\text{CaCl}_2$ solution (10 mM each). The mixture was incubated at 37°C for 10 minutes and then inoculated into 400 ml of NZCYM broth (NZY broth plus 1 g/l Casamino acids) and incubated at 37°C with shaking overnight until phages lysed. 0.8 ml chloroform was added into the flask, and then incubated at 37°C for further 15 minutes. The cell debris were spun down at 7,000 rpm for 10 minutes at 4°C in chloroform-resistant 500 ml-bucket, the supernatant was cleaned by filtration and transferred into a 1 liter-glass beaker, and 23.4 g NaCl and 40 g polyethyleneglycol (PEG 8,000) were added. After shaking at 37°C until complete dissolving of PEG, the beaker was placed in an ice-water bath for about 45 minutes to precipitate phages. Phages were then collected by centrifugation at 7,000 rpm for 30 minutes at 4°C . The phage pellet was resuspended in 6.5 ml 1x buffer A (freshly prepared by mixing of 5 ml 10x Buffer A, 45 ml H_2O and 20.5 μl 14.63 M 2-mercaptoethanol). Phages were harvested as much as possible into a 50 ml-Falcon tube, incubated with 16 μl DNase I (10 mg/ml) and 40 μl RNase A (10 mg/ml, heat-inactivated) for 30 minutes at 30°C . Removal of PEG was performed by extraction once with an equal volume of chloroform, the mixture was shaken gently for 5 minutes and spun at 3,000 rpm for 10 minutes at 20°C . The upper phage layer was carefully collected. In order to purify the phage DNA, an one-step gradient in conical polyallomer 10 ml-tube (14x89 mm, Beckmann, USA) was set up with the bottom layer of 0.75 ml 40% glycerol/1x Buffer B/6 mM 2-mercaptoethanol, the middle layer of 2.5 ml 5% glycerol/1x Buffer B/ 6 mM 2-mercaptoethanol and upper layer of 8 ml phage solution, and centrifuged at 35,000 rpm for 1 hour at 4°C with L8-70M Ultracentrifuge (Beckman, USA). The supernatant was carefully discarded, the phage pellet was resuspended into 1.6 ml of 2x ET buffer (40 mM Tris-HCl, pH7.5/0.1 M EDTA) and transferred into a 5 ml-cryotube (Nunc,

Germany). After adding of 0.4 ml of 10% SDS, the suspension was incubated overnight at 55°C. Phage DNA was then extracted with an equal volume of phenol, phenol/chloroform and chloroform by gentle inversion and centrifugation at 2,000 rpm for 5 minutes at RT, respectively. Afterwards, the upper phase was carefully transferred into a fresh 15 ml-Falcon tube. Phage DNA was precipitated by mixing well with 2 volume of 100% ethanol at RT. The visible DNA clump was pulled out with a bended glass capillary and briefly washed by dipping the clump 5-10 times in 70% ethanol, then dissolved into 500 µl of 0.1x TE buffer. The phage DNA solution was stored at -20°C until use.

2.2.6.7 Sequence analysis

After restriction mapping, subcloning and sequencing, the genomic *CBP* was analysed by using HUSAR program (Heidelberg Unix Sequence Analysis Resources, German Cancer Research Center, DKFZ, Germany).

2.2.7 Embryonic stem (ES) cell culture

2.2.7.1 Preparation of mouse fibroblast feeder cells

G418-resistant fibroblast feeder cells were isolated from the G418-resistant C57BL/6 J-TgN (PGK-Neo bpA) 3-Ems transgenic mouse strain, and purchased from The Jackson Lab., USA. The G418-resistant feeder plates were prepared same as the commonly used feeder plates described below.

Primary embryonic fibroblast cells were isolated from mouse embryos at 14-16 days post coitus (dpc). The pregnant mouse was killed, the uterus was isolated and placed in a 10 cm Petri dish containing PBS. The embryos were dissected from the uterus free of

all the extraembryonic membranes, then transferred into a new dish containing PBS and heads were removed. The embryos were transferred into dishes without PBS, all internal organs (liver, heart, kidney, lung and intestine) were removed, about 10 carcasses were isolated in this way and put in new dishes containing PBS. The carcasses were then washed three times in a 50 ml Falcon tube with 35 ml PBS to remove as much blood as possible. After sucking off the PBS, the carcasses were transferred into a Petri dish (on ice) and quickly minced with a sterile scalpel. The minced bodies were collected in 10 ml of 0.25% trypsin/EDTA solution (GIBCO, Germany) supplemented with 200 µg/ml of DNase I, and incubated for 15 minutes at 37°C. A mesh was sterilized in boiling water for 30 minutes, then cooled down to RT, washed with PBS and 0.25% trypsin/EDTA solution and placed on an Erlenmeyer flask. The minced bodies were pressed with the syringe plunger without needle through the mesh. A total about 50 ml 0.25% trypsin/EDTA solution was used to flush the mesh. The elution was incubated with shaking for 30-45 minutes at 37°C. The cell suspension was decanted in two 50 ml Falcon tubes, filled up with feeder DMEM medium (same as E14 complete medium, but without LIF) and spun down at 1,500 rpm for 5 minutes. The cell pellet was washed at least twice with DMEM, and then resuspended in 10 ml feeder DMEM medium, and viable cells were counted using Trypan blue method (Sigma, Germany).

5×10^6 viable cells were plated onto 15 cm-dish containing 25 ml feeder DMEM medium and were incubated at 37°C, 5% CO₂ overnight, the next day changed with 25 ml of fresh feeder DMEM medium. 2 or 3 days later, the fibroblast cells were trypsinized and split 1:5 onto new dishes for further incubation. After another 2 or 3 days, cells were confluent and to be frozen at -80°C (about $5-10 \times 10^6$ cells/vial), then stored in liquid nitrogen.

One vial of frozen primary embryonic fibroblast cells was quickly thawed at 37°C and transferred into a 15 ml Falcon tube containing 10 ml feeder DMEM medium. After centrifugation at 1,200 rpm for 5 minutes at RT, the cells were resuspended into 5 ml feeder DMEM medium. The cell suspension was distributed evenly onto five 15 cm-dishes each containing 25 ml feeder DMEM medium and incubated at 37°C, 5% CO₂ until cells reaching a confluent mono-layer (about 4 days). The cells were trypsinized and transferred in Falcon tubes. After centrifugation, the cell pellet was resuspended into feeder DMEM medium. The cell suspension was then distributed evenly onto twenty 15 cm-dishes and incubated for 3 days, then treated with feeder DMEM medium containing 0.01 mg/ml mitomycin C (stock one: 1 mg/ml in PBS, stored in dark at 4°C and used within two weeks) for 3 hours at 37°C, 5% CO₂. Lastly, cells were trypsinized as usual and plated onto convenient dishes at the concentration of 1×10^4 cells/cm². The feeder plates were then fed with feeder DMEM medium, and stored at 37°C, 5% CO₂ up to 7 days.

2.2.7.2 ES cell culture

E14 mouse embryonic stem cells originally isolated from inbred blastocysts of 129/Ola strain (Hooper *et al.*, 1987), were kindly provided by Dr. Klaus Pfeffer, Department of Immunology, Technical University of Munich, Germany.

2.2.7.2.1 Trypsinization of ES cells

Trypsinization was performed as following: cells were washed once with 1x PBS (without Ca²⁺ and Mg²⁺, GIBCO, Germany). After aspiration of PBS, ES cells were mixed with proper amount of 0.25% trypsin/EDTA solution (Tab. 2.22) and incubated at 37°C, 5% CO₂ for 15 minutes. Cell suspension was transferred into a 15 ml Falcon

tube containing 10 ml E14 complete medium (see Tab. 2.5). After centrifugation at 1,200 rpm for 4 minutes at RT, cell pellet was resuspended in the medium and was ready for further expansion, or other purposes.

Table 2.20 Parameters of cell culture plates or dishes

Dish and plate	Area (cm ²)	Amount of medium (ml)	Amount of trypsin (ml)
15 cm-dish	145	25	7.5
10 cm-dish	56.7	10	4
6 cm-dish	20.8	6	2
6 well-plate	8.7	2.5	0.5
24 well-plate	1.9	1	0.15
96 well-plate	0.33	0.2	0.05

2.2.7.2.2 Thawing and expanding of ES cells

A vial of E14 ES cells (approx. $1\text{--}2 \times 10^6$ cells) was quickly thawed in a 37°C water bath, then transferred into a 15 ml Falcon tube containing 10 ml of E14 complete medium and mixed gently by inversion. After centrifugation at 1,200 rpm for 5 minutes at RT, the cell pellet was resuspended in 6 ml E14 complete medium, then plated onto a 6 cm-feeder dish, and incubated at 37°C, 5% CO₂ overnight. The next day, the cells were washed once with 1x PBS, fed with 6 ml of fresh E14 complete medium and incubated at 37°C, 5% CO₂ for another day. The cells were trypsinized and plated in an approximately 1:3 dilution onto a 10 cm-feeder dish, incubated at 37°C, 5% CO₂ for another 2 days. The cells were split again onto three 10 cm-feeder dishes for further expansion on gelatinized dishes (gelatin pre-treatment of dishes was performed by incubation with 0.1% gelatin for a few minutes. Afterwards, gelatin solution was

aspirated, and dishes were filled with E14 complete medium.) for another 2 days. ES cells were then ready for electroporation.

2.2.7.2.3 Electroporation of ES cells, antibiotic selection, picking, expansion and freezing of cell clones

Electroporation of ES cells. E14 ES cells on gelatinized dishes were trypsinized and resuspended in PBS. After counting (Neubauer improved cell count slide, Marienfeld, Germany), 7×10^6 cells in 800 μ l cold PBS of E14 cell suspension was prepared and transferred into one 0.4 cm electrode gap gene pulser cuvette. Targeting constructs were linearized by digestion with proper restriction enzymes (see Construction of targeting vectors in Chapter 4) for overnight. Complete linearization of targeting constructs was checked by agarose gel electrophoresis. The enzymes were then heat-inactivated at 70°C for 20 minutes. 20 μ g in a volume of each linearized DNA was added into the cuvette. Electroporation was performed with the conditions of 240 V, 500 μ F for about 6.0 milliseconds with a BioRad Gene Pluser. The cuvette was then incubated for 15 minutes on ice. The cell suspension was distributed evenly onto two 10 cm-feeder dishes (or G418 resistant feeder dishes if Neo selection was performed) each containing 10 ml of E14 complete medium. ES cells were incubated at 37°C, 5% CO₂ overnight.

Antibiotic selection. The day after electroporation, the medium was substituted with selection medium. When Neo selection gene was used, the cells were daily changed with E14 complete medium supplemented with 200 μ g/ml geneticin G418 until formation of ES cell colonies ready for picking. When thymidine kinase gene was used, ES cells were daily changed with freshly prepared E14 complete medium supplemented with 2 μ M gancyclovir for 2 days, then daily changed with E14 complete medium without gancyclovir until the formation of ES cell colonies ready for picking. When

puromycin resistant gene was used, selection was performed for the first two days with 1 µg/ml puromycin in E14 complete medium. Then, the cells were daily fed with fresh E14 complete medium without puromycin until the formation of ES cell colonies for picking.

Picking of single ES clones. Generally, after 8-9 days of selection and growth of ES cells, ES cell colonies were visible and ready for picking. The cells were fed with fresh E14 complete medium, and the colonies were picked with 200 µl aerosol resistant filter tips (USA) under the microscope (Leica MZ6, Germany) into 96 well-feeder plates containing fresh E14 complete medium without any selection antibiotics, and the plates were then incubated at 37°C, 5% CO₂ overnight.

Expansion of ES clones. One day after picking, “tryplation” was performed. Each cell clone was washed once with 1x PBS and trypsinized with 50 µl of 0.25% trypsin/EDTA at 37°C, 5% CO₂ for 15 minutes. Then 150 µl of E14 complete medium was added to stop trypsinization. The cell clumps were pipetted up and down carefully to get single cells in the original 96 well-feeder plates, and cells were then incubated at 37°C, 5% CO₂ for two days. Principally, ES cells have to be trypsinized every two-days, otherwise they might start to differentiate. For slower growing clones, trypsinization was performed every two days until reaching proper density. Finally, the ES cells were split into three 96 well-feeder plates and incubated at 37°C, 5% CO₂. Two days later, the cells of one of the 96 well-feeder plate were split again into three 96 well-gelatinized plates, incubated for another two days or until reaching confluency. They were then ready for genomic DNA extraction and later for Southern blot analysis. The cells of the two other feeder plates were be frozen.

Freezing of ES clones. For freezing of ES cells in 96 well-plates, ES cells were firstly trypsinized as usual with 50 μ l of 0.25% trypsin/EDTA at 37°C, 5% CO₂ for 15 minutes. Then, 50 μ l of E14 complete medium was added to stop trypsinization, and cells were mixed well by pipetting up and down with 100 μ l of cold 2x freezing medium. The plates were sealed with Parafilm (American National Can™, USA) and frozen immediately at –80°C up to three months.

Expansion of positive clones. After the positive clones were examined by Southern blot and/or PCR, the corresponding frozen clones in 96 well-feeder plates were thawed quickly in a 37°C water bath, and expanded progressively on feeder cells in the order of 96 well-plates, 24 well-plates, 6 well-plates, 6 cm-dishes and finally 10 cm-dishes. The cells on 10 cm-feeder dishes were trypsinized and resuspended in 2 ml of E14 complete medium, then, evenly distributed into four 2 ml-freezing vials (Nalgene, UK). 0.5 ml of ES cell suspension/vial was added and mixed well with an equal volume of pre-cold 2x freezing medium. The vials were immediately transferred into pre-cooled Cryo 1°C Freezing Container (Nalgene, UK), stored at –80°C for a few days and then transferred into liquid nitrogen until use.

2.2.8 Blastocyst injection

Blastocyst injection was performed as described by Hogan *et al.* (1994), including superovulation by hormone injection using pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG), glass microinstrument fabrication, blastocyst collection, identification of pseudopregnant females, surgical transfer of blastocysts to pseudopregnant recipients, and microinjection of ES cells into the blastocoeles of C57BL/6 blastocysts.

2.2.9 Examination of Cre-mediated inversion in ES cells

One of the correct homologously recombined ES cell lines (F3B12) from targeting vector CBP^{Tyr658Ala} was expanded and finally, 1×10^6 ES cells in 800 μ l PBS buffer were prepared and transferred into the electroporation cuvette (Gene Pluser Cuvette 0.4 cm electrode gap, BioRad Laboratories, USA), mixed with 20 μ g pCre-pac plasmid which contains the genes for Cre recombinase and puromycin resistancy driven under constitutive promoters. After electroporation (240 V, 500 μ F), the cuvette was incubated for 15 minutes on ice. Cell suspension was then evenly distributed onto three 10 cm-feeder dishes, fed with DMEM (Dulbeccos' Modified Eagles Medium, high glucose, plus Na-Pyruvate) E14 complete medium and incubated at 37°C, 5% CO₂ overnight. The cells were then daily fed for two days with fresh E14 complete medium supplemented with 1 μ g/ml puromycin for selection of Cre recombinase expression, then daily fed with E14 complete medium until formation of ES colonies. 96 single colonies were picked and expanded, from where two batches of cells on 96-well feeder plates were frozen at -80°C and three batches of cells were expanded on 96-well gelatinized plates for Southern blot analysis.

Chapter 3

Results

3.1 Expression pattern of CBP

3.1.1 Introduction

CBP is expected to be expressed in most, if not all, tissues, due to its characteristics as a multifunctional molecular integrator of many signalling pathways. As outlined in Chapter 1, the physiological roles of CBP in the cAMP-CREB pathway were to be investigated, for example, in the context of learning and memory. An immunohistological study showed that CBP is expressed in the nuclei of a large number of neurons and glial structures in rat brain and spinal cord. Intense CBP-labelling was found, among others, in the hippocampus, amygdala and the thalamus, suggesting that CBP may be involved in the formation of long-term memory and modulation of cortical activities (Strömberg *et al.*, 1999). However, the expression pattern of CBP in the adult mouse brain has not yet been characterized. As the examination of the expression of CBP at a single cell resolution is a prerequisite for better understanding of the physiological functions of CBP in mouse, *in situ* hybridization and immunohistochemistry experiments were performed.

3.1.2 Preparation of riboprobes

To determine the expression patterns of CBP and p300, both radioactively (^{35}S) and non-radioactively (digoxigenin) labelled riboprobes were used for *in situ* hybridization experiments. The templates were generated by RT-PCR from cDNA derived from total mouse brain RNA. The primers were synthesized based on the cDNA sequence

deposited in GenBank (accession number S66385 for mouse *CBP*, and AF000581 for mouse *p300*). The sequence of the CREB-binding domain (CBD) from *CBP* and the related sequence from *p300* were amplified by PCR with primers Z3-Z4 and Z7-Z8 (see Table 2.6), respectively, then subcloned into plasmid pBSII KS (-) and used as templates for riboprobe synthesis. The sequences were confirmed by sequencing. The generation of sense and antisense riboprobes for *CBP* and *p300* are described in Table 3.1 and were made by *in vitro* transcription.

Table 3.1

Plasmid	probe	Enzyme for linearization / RNA polymerase
pBS-CBD.CBP	Antisense	<i>Hind</i> III / T7
	Sense	<i>Eco</i> R I / T3
pBS-CBD.p300	Antisense	<i>Eco</i> R I / T3
	Sense	<i>Hind</i> III / T7

The riboprobes for *CBP* and *p300* (including antisense and sense probes) were labelled with ^{35}S -UTP or digoxigenin-labelled UTP. The integrity of DIG probes was determined by 1.0% agarose gel electrophoresis, and the quantitative analysis was achieved by using DIG Quantitation and DIG Control Teststrips.

3.1.3 *In situ* hybridization with radioactive probes

In situ hybridization experiments on mouse brain sections derived from adult wild-type C57BL/6 mice revealed that both *CBP* and *p300* were highly expressed in most of areas of the forebrain, including different layers of cortex, hippocampal CA1 and CA3 regions and dentate gyrus, entorhinal cortex, amygdala and various thalamic nuclei (Fig. 3.1). Sections hybridized with sense control probes showed no signal.

In situ hybridization

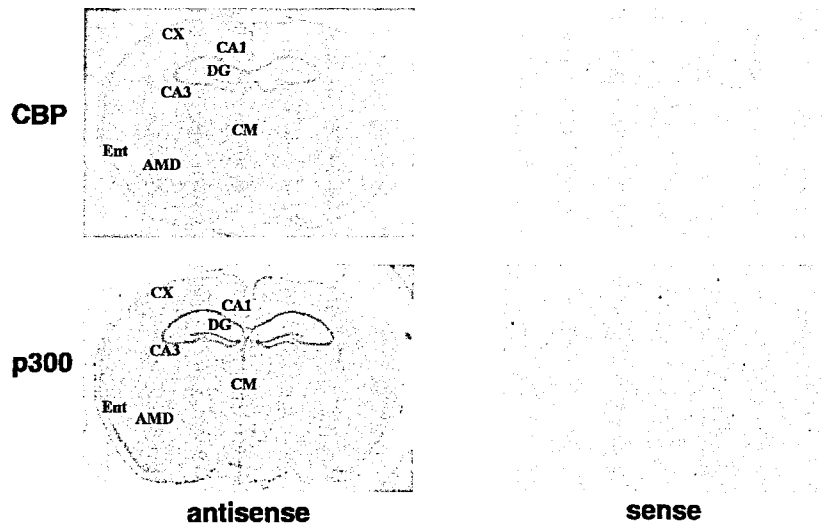


Fig. 3.1: Expression pattern of CBP and its related protein p300 using ^{35}S -labelled riboprobes. Both genes are strongly expressed in neocortex (CX), hippocampus (CA1, CA3 and DG), entorhinal cortex (Ent), amygdaloid (AMD) and central medial thalamic (CM) areas. Control hybridization with sense probes did not show any signal.

3.1.4 *In situ* hybridization with non-radioactive probes

As the hybridization signals obtained with radioactive probes were rather low in intensity, it was not possible to evaluate the expression pattern at a single cell resolution. Thus, digoxigenin-labelled CBP riboprobes were used for *in situ* hybridization on wild-type C57BL/6 adult mouse brain sections. As shown in Fig. 3.2, CBP is expressed in most of the neurons in cerebral cortex, hippocampal CA1, CA3 pyramidal layers (pyl) and dentate gyrus (DG), but remarkably expression is not ubiquitous, e.g. many interneurons in the hippocampal formation do not express CBP.

Cells lacking CBP transcripts were not only found in hippocampal areas, but also in the neocortex. Different intensities of CBP expression levels were found in the different layers of the cortex (Fig. 3.2A); layers I, II, III, V and VI showed strong expression

levels of CBP, but layer IV, comprising mostly granular cells, showed only sparse CBP expression. Many cells were also found negative for CBP in white matter (WM), hippocampal polymorphic (pl) and molecular layers (ml) (Fig. 3.2C), and all three layers of the dentate gyrus (Fig. 3.2D).

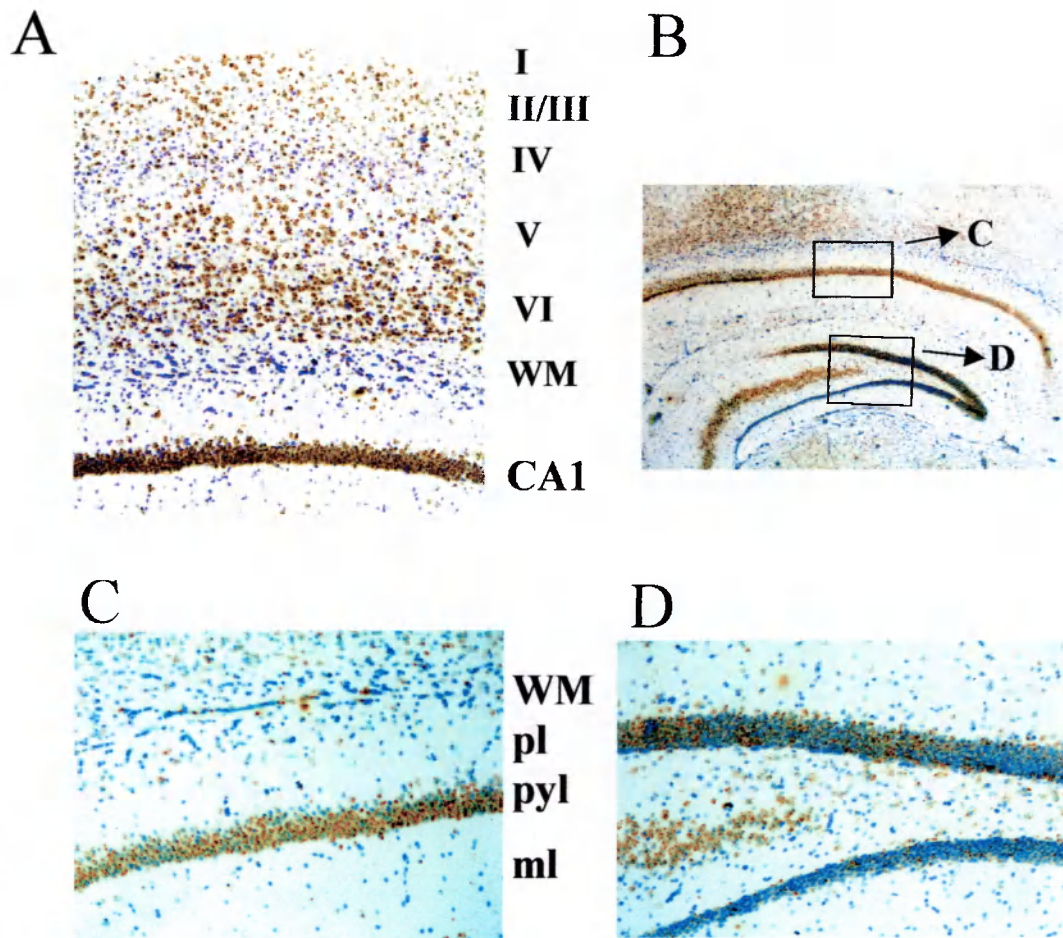


Fig. 3.2: Expression pattern of CBP using DIG riboprobe (brown staining) in cortex (visual cortex) and hippocampus. All sections were counterstained with toluidine blue. A. CBP is strongly expressed in most layers of cortex, but layer IV and white matter (WM) show sparse CBP expression intensities. B. CBP appears to be highly expressed in most hippocampal areas. C, D: Higher magnification of areas indicated in B, showing CBP expression in three layers of hippocampal CA1 areas (C) and dentate gyrus (D), respectively. pl: polymorphic layer, pyl: pyramidal layer, ml: molecular layer.

CBP expression was further examined in other regions of the limbic system. Both entorhinal cortex and amygdala showed rather strong expression levels (Fig. 3.3), where only very few CBP-negative cells were detected .

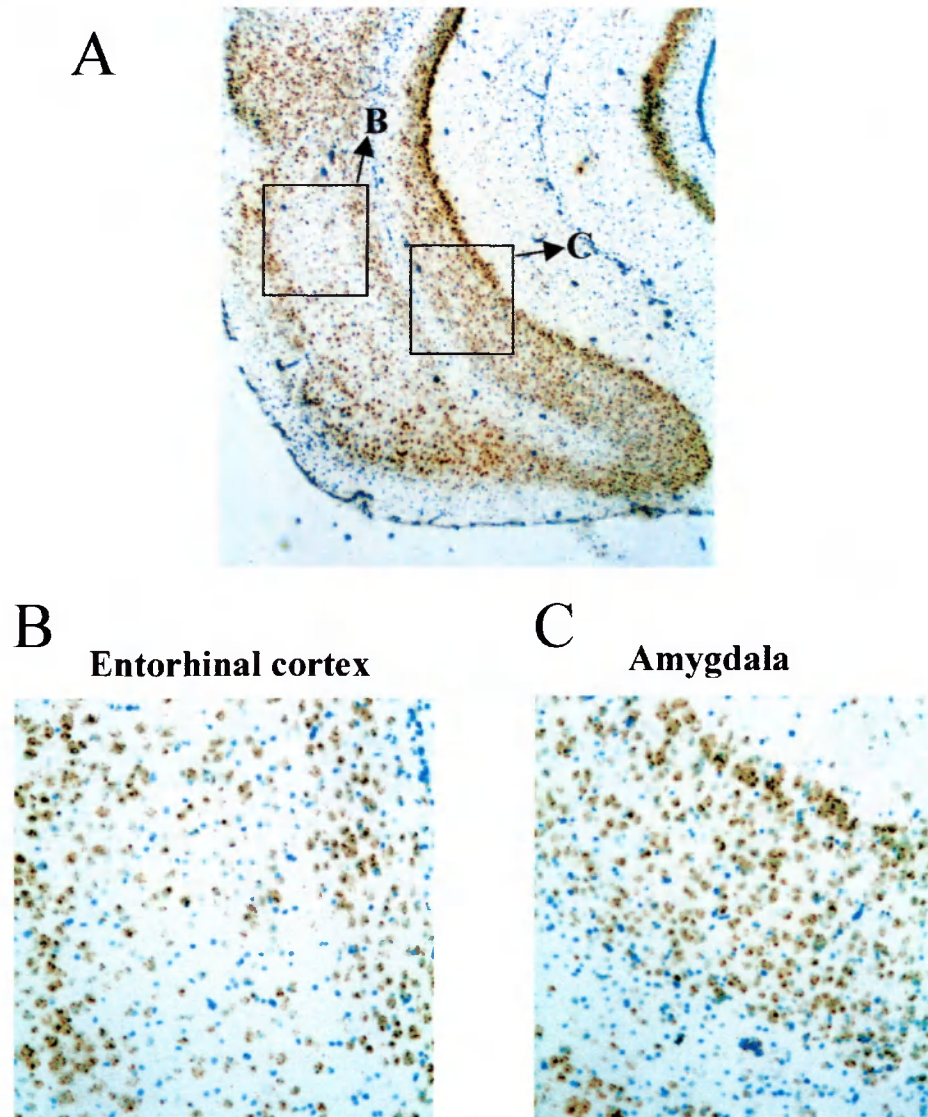


Fig. 3.3: Expression pattern of CBP using DIG riboprobe (brown staining) in entorhinal cortex and amygdala. Sections were counterstained with toluidine blue. A. Both entorhinal cortex and amygdala areas showed strong expression level of CBP. B, C. Higher magnification of areas indicated in A, showing CBP-labelling in entorhinal cortex (B) and amygdala (C), respectively.

3.1.5 Immunostaining

The expression of CBP at the protein level was examined by immunostaining with a monoclonal antibody to the C-terminus of CBP (Santa Crus, C-1) on paraffin sections of adult mouse brain. All sections were counterstained with toluidine blue. Intense CBP labelling was found in most of the neocortex and in most principal neurons of hippocampal CA1/CA3 areas and granular cells of dentate gyrus. Few of the cells, however, were CBP-negative in the visual cortex (Fig. 3.4A, B), and hippocampal CA1 and DG areas (Fig. 3.5A, B).

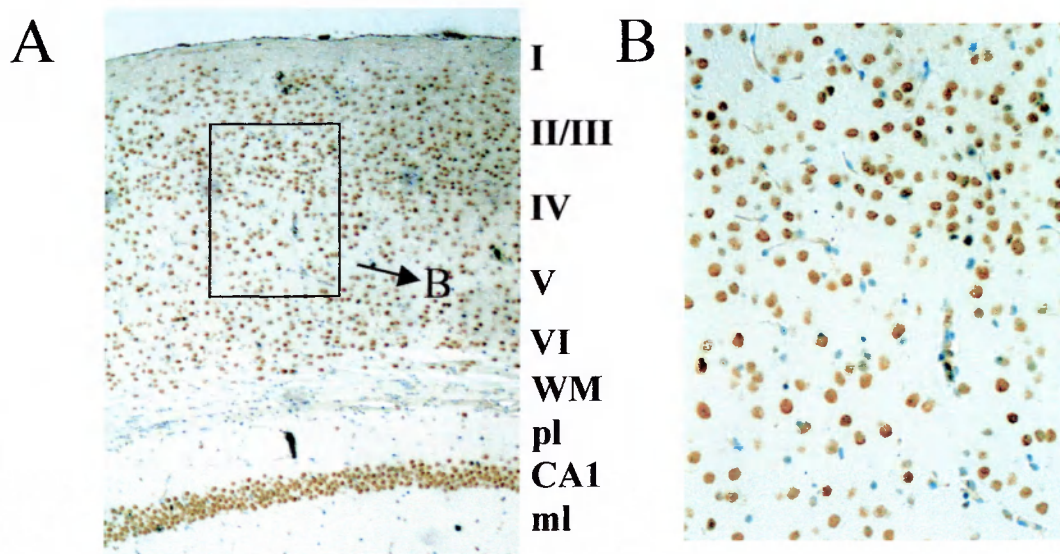


Fig. 3.4: Immunostaining with anti-CBP antibody (brown staining) in visual cortex and hippocampal CA1 areas. Sections were counterstained with toluidine blue. A. CBP appears to be highly expressed in most of the neocortex and hippocampal CA1 areas. B. Higher magnification of areas indicated in A, showing CBP expression in neocortex. Note that CBP is not ubiquitously expressed in cortex: the highest frequency of negative cells is found in layer IV (B). I to VI: cortex layers, WM: white matter, pl: polymorphic layer, ml: molecular layer.

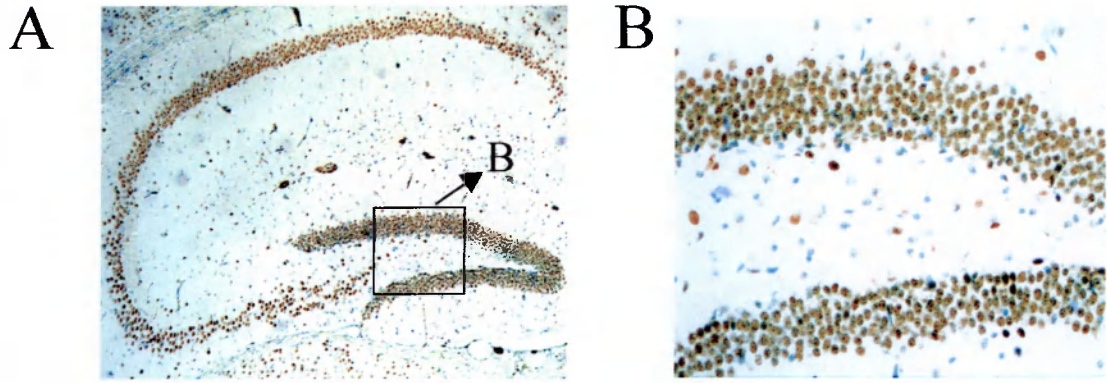


Fig. 3.5: Immunostaining with anti-CBP antibody (brown staining) in hippocampal CA1/CA3 and DG areas. Sections were counterstained with toluidine blue. A. CBP appears to be highly expressed in most of the hippocampus. B. Higher magnification of areas indicated in A, showing CBP expression in dentate gyrus. Note that CBP is not ubiquitously expressed in dentate gyrus (B).

Immunostaining was further performed in other regions of the limbic system, such as entorhinal cortex (Fig. 3.6) and amygdala (Fig. 3.7), where CBP was found to be present in many cells.

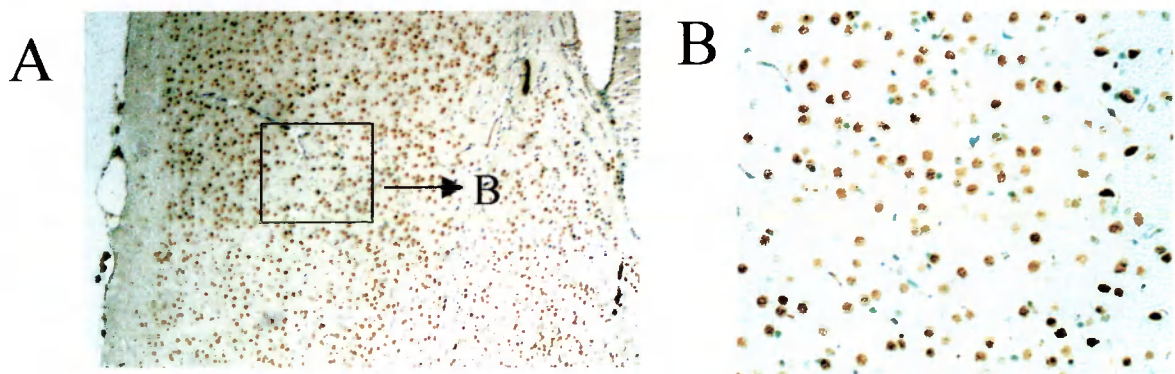


Fig. 3.6: Immunostaining with anti-CBP antibody (brown staining) in entorhinal cortex. Sections were counterstained with toluidine blue. A. CBP appears to be highly expressed in entorhinal cortex. B. Higher magnification of areas indicated in A, showing that CBP expression is not ubiquitous. Few of CBP-negative cells were found in entorhinal cortex.

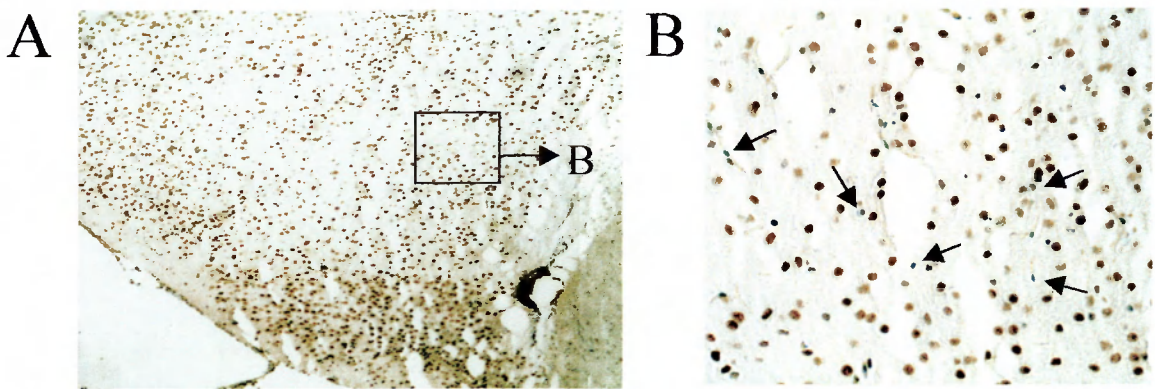


Fig. 3.7: Immunostaining with anti-CBP antibody (brown staining) in amygdala. Sections were counterstained with toluidine blue. A. CBP appears to be highly expressed in amygdala. B. Higher magnification of areas indicated in A, showing that CBP expression is not ubiquitous: few CBP-negative cells indicated by arrows were found in amygdala.

In conclusion, both CBP and p300 are present in most of neuronal population in mouse forebrain, i.e. neocortex, hippocampus, entorhinal cortex and amygdala, and central medial thalamic areas. However, CBP expression is not ubiquitous as predicted, even in the forebrain region. Many CBP-negative cells were found in the dentate gyrus, cortical layer IV and few cells in hippocampal CA1 and CA3 areas. CBP expression pattern in protein level detected by Immunostaining was highly correlated with it in mRNA level.

3.2 Mutational analysis of CBP *in vitro*

Six CBP mutants were proposed for mutational analysis *in vitro* (Fig. 3.8). Three of them were generated in order to know whether it is possible to interfere with cAMP signalling by removing only part or all of the CBD, but to keep intact other functions of CBP, such as steroid hormone receptor-mediated transcriptional regulation. The deletion mutation CBP Δ CBD2-5 lacks amino acids 524 to 704 (almost the whole CBD); CBP658Ala is a point mutation at residue 658, which fully abolishes the CREB-binding

activity of CBP *in vitro* (Radhakrishnan *et al.*, 1997); lastly, CBP1100aa retains only the N-terminal 1100 amino acid residues of CBP, including the whole CBD. Another three mutants are so-called RTS mutants: RTS1 and RTS2 are CBP mutants that retain only the N-terminal 136 and 357 amino acid residues, respectively. Both correspond to the CBP mutations found in RTS patients (Petrij *et al.*, 1995). RTS3 is a longer truncated CBP mutant that retains the N-terminal 523 amino acid residues, including the N-terminal transactivation domain of CBP, but lacking most of the CREB-binding domain.

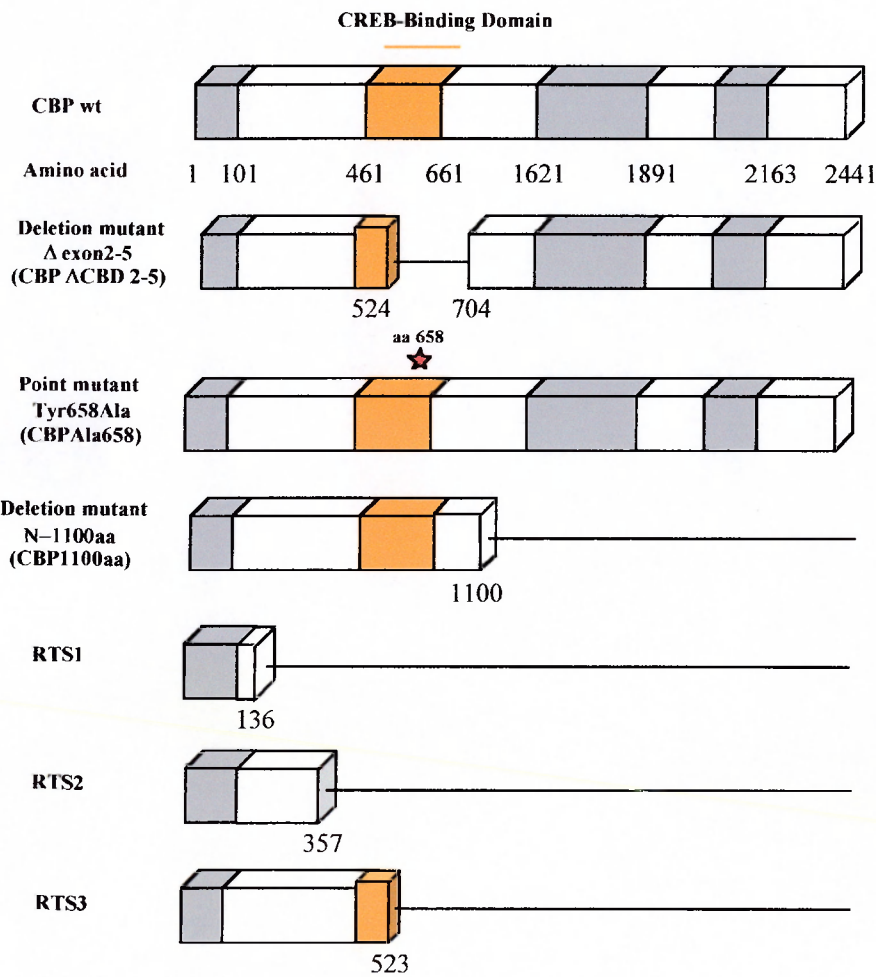


Fig. 3.8: Schematic representation of six CBP mutants. The CREB-binding domain is marked in yellow. Amino acid residue 658 in CBD exon 5 is indicated with asterisk. The grey and white areas are different binding domain to many other proteins (for further details, see Fig. 1.5).

Wild-type CBP or these mutants, together with either CRE-luciferase or GRE-luciferase reporter constructs were co-transfected into F9 cells, and the luciferase activities induced by PKA or dexamethasone, respectively, were measured. The mutants that lack an intact CBD should also lack PKA-induced transcriptional enhancement through CRE-luciferase, but retain dexamethasone-induced enhancement through GRE-luciferase. By these experiments, it should be able to define which part of CBP is essential for its transcriptional activation through the cAMP pathway, and provide an evidence how to generate a CBP knock-out mouse which lacks its cAMP-mediated activation potential, but retains other functions mediated by other transcription factors, this mutant mouse could let us get insight into its functions through cAMP-CREB pathway.

3.2.1 Generation of CBP mutants

CBP Δ CBD2-5: a deletion mutation, in which the CREB-binding domain (CBD) encoded by CBD exons 2 to 5 was deleted by PCR, without affecting the rest of the CBP coding region. Amino acid residues 524-704 are encoded by these exons, and comprise almost the entire CBD of CBP. The whole cDNA clone of mouse CBP (pRc/RSV-mCBP.HA) (Fig. 3.9) was used as template to amplify the fragment containing sequence nt 1556-1570 + nt 2111-3374 (for cDNA sequence and coordinates, see Appendix I) with Pfu polymerase and primers Z15-Z16 (see Table 2.6). Z15 is a 37-nucleotides forward primer, containing 17 nucleotides (nt 1556-1570 + GTA) from the 3' end of CBD exon 1 including one endogenous *Avr*II site, and another 20 nucleotides (nt 2111-2130) located at the very 5' end of CBD exon 6. There is another *Avr*II site located on the reverse primer Z16 (nt 3345-3374). Thus, the Z15-Z16 PCR fragment is flanked by two *Avr*II sites. After purification of the PCR product

with QIAquick PCR purification kit, it was digested by *AvrII* at both ends. The DNA fragment Z15-Z16/*AvrII* was subcloned into pBSIIKS plasmid, which had first been digested by *AvrII* and then dephosphorylated with shrimp alkaline phosphatase (SAP). The final clone was confirmed by restriction digestion and sequencing. The whole insert from the correct clone was the subcloned into the expression vector pRc/RSV.

CBPAla658: a point mutation, tyrosine 658 was changed into alanine by overlapping PCR with two pairs of primers, Z19-Z20 and Z21-Z18. The fragment with the point mutation was obtained by PCR with primers Z19 and Z18, and both Z19-Z20 and Z21-Z18 PCR products were used as further templates. Firstly, the whole cDNA clone of mouse CBP (pRc/RSV-mCBP.HA, see Fig. 3.9) was used as template to amplify two fragments, nt 1637-1984 and nt 1960-3307, with primers Z19-Z20 and Z21-Z18, respectively. There are 24 nucleotides of overlap between the two PCR fragments. Concomitant with the point mutation Tyr658Ala generated by primers Z20 and Z21, a new unique *NruI* restriction site was introduced. The original unique sites *SapI* and *XbaI* are located in primers Z19 and Z18, respectively. Both fragments Z19-Z20 and Z21-Z18 were then used as templates to amplify the fragment nt 1556-3374 including the unique sites the *SapI* and *XbaI* at both ends with primers Z19-Z18. The PCR product Z19-Z18 was purified by QIAquick PCR Purification Kit and digested by *SapI* and *XbaI*. The DNA fragment Z19-Z18/*SapI-XbaI* was subcloned into pBSIIKS vector, which was firstly digested by *XbaI* and then partially digested by *SapI*, because there is one additional *SapI* site in the vector backbone. The sequence of the final clone was confirmed by restriction digestion and sequencing. The whole insert from the correct clone was the subcloned into the expression vector pRc/RSV.

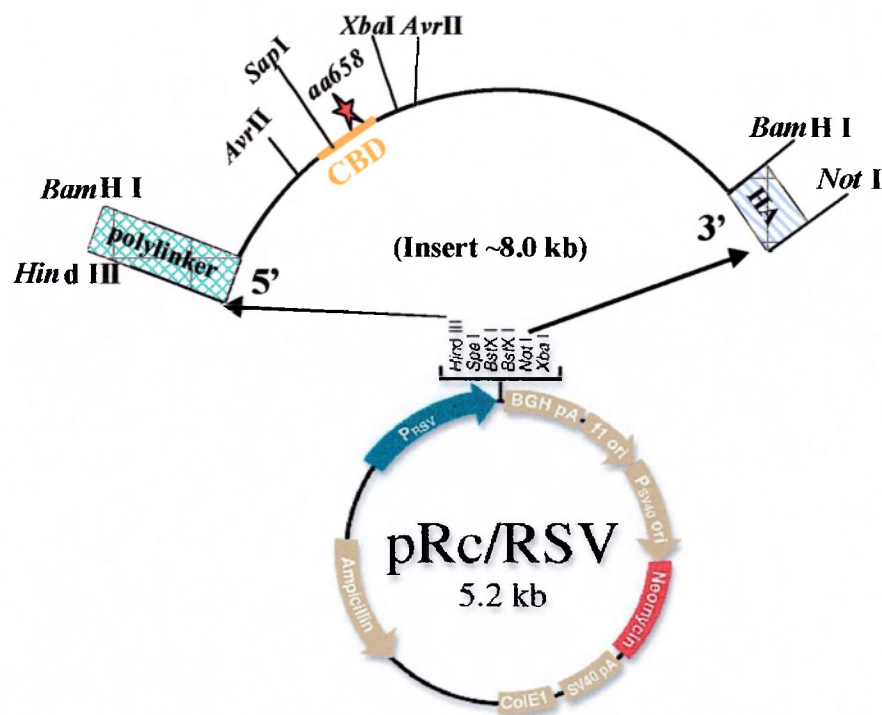


Fig. 3.9: Restriction map of pRc/RSV-mCBP.HA, including whole cDNA of mouse CBP with the HA (hemagglutinin) tag at its 3' end. The CBD, amino acid residue 658 and useful restriction sites are indicated.

CBP1100aa: CBP1100aa was generated by deletion of the whole C-terminus of CBP downstream from the position of a unique restriction site *XbaI* at nt 3300. In order to keep the HA tag at the 3' end of the mouse cDNA clone in frame (referring to Fig. 3.9), mung bean nuclease and T4 polymerase were used for removal and fill-in of the 5' overhang after *BamHI* and *XbaI* digestions, respectively. There are two *XbaI* sites in the original plasmid pRc/RSV-mCBP.HA, one is located at nt 3300 (amino acid residue 1100), another in the 3' polylinker region; two *BamHI* sites are present, one is in the 5' polylinker region and another between the *CBP* cDNA and the HA (hemagglutinin) tag; one *HindIII* site is also located in the 5' polylinker region. To generate CBP1100aa, which encodes only the N-terminal 1100 amino acid residues and the HA tag, pRc/RSV-mCBP.HA was digested with *BamHI*, and the 5.2 kb vector backbone was isolated by agarose gel electrophoresis. The fragment was treated with mung bean

nuclease to remove the 5' overhang, and then digested with *HindIII*. The vector backbone with one *HindIII* sticky end and one modified *BamHI* blunt end was purified. For the insert, pRc/RSV-mCBP.HA was digested with *XbaI*, and the fragment containing the vector and the 5' end of CBP was purified by agarose gel electrophoresis, and then treated with T4 polymerase to fill in the 5' overhang. After purification, the fragment was digested with *HindIII*. The 3.0 kb 5' CBP fragment with one *HindIII* sticky end and one modified *XbaI* blunt-end was isolated by agarose gel electrophoresis and purified by QIAGEN Gel Purification Kit. After ligation of this fragment to the vector fragment with a *HindIII* sticky end and a *BamHI* modified blunt end and recovery of colonies in *E. coli*, the sequence of mutant CBP1100aa was confirmed by restriction digestion and sequencing.

RTS mutants: three RTS mutants were generated by PCR using the original cDNA clone of CBP as template. Forward primer Z29 was common to all three mutants, which is located approximately at the start codon of the cDNA (see Fig. 3.9), and includes a *HindIII* restriction site located in polylinker. Reverse primers Z30, Z31 and Z32 (referring to Table 2.6 for the sequence and location) were used for amplification of *CBP* RTS1, RTS2 and RTS3 fragments, respectively (see Fig. 3.8 for positions of truncations), together with primer Z29. A unique *NotI* site and a stop codon were included in the 5' end of these three primers, respectively. After PCR with Pfu polymerase, the three PCR products were digested with *HindIII* and *NotI*, and then subcloned into the expression vector pRc/RSV. The correct clones were confirmed by restriction digestion and sequencing.

Several errors were found in the GenBank cDNA sequence of CBP (accession number S66385) by sequencing of the cDNA plasmid clone pRc/RSV-mCBP.HA during the mutagenesis experiment. Thymidine (T) at nucleotide 2008, 2477, 2871 and 2932 of

the mouse cDNA clone were detected instead of adenosine (A) in the published mouse cDNA sequence. Interestingly, all these discrepancies in mouse cDNA were replacement of the corresponding human cDNA sequence of *CBP* gene.

3.2.2 Transfection assays

PKA activity is increased upon differentiation of F9 embryonal carcinoma (EC) cells, but cannot be activated by cAMP in undifferentiated F9 cells, although PKA is expressed at the protein level both by undifferentiated and differentiated F9 cells (Kingsley-Kallesen *et al.*, 1999). Furthermore, the Ser¹³³-phosphorylated form of CREB is present in the nucleus of differentiated F9 cells, but is not detected in the nucleus of undifferentiated F9 cells. Thus, the differentiated F9 cells were used to test the functions of CBP *in vitro*.

CBP wild-type and two CBP mutants (CBPA1a658 and CBPΔCBD2-5, respectively) were separately cotransfected into differentiated F9 cells together with pHD-CREB, pCEV (catalytic unit of PKA) and reporter construct CRE (CREB responsive element)-luciferase. Two days later, luciferase activity was measured. Both mutants significantly interfered with the cAMP-regulated transcription (Fig. 3.10A). Relative to levels of reporter gene expression obtained using 1 µg CBP, 73% CRE-luciferase activity was observed without any CBP added, 45% activity when the CBPA1a658 mutant was used instead of wild-type CBP, and 50% activity when the CBPΔCBD2-5 mutant was used instead of wild-type CBP. This suggests that both mutants have a dominant-negative effect on cAMP signalling. This effect of CBPA1a658 was dose-dependent (Fig. 3.10B). Increasing the ratio of CBPA1a658 to wild-type CBP led to a concomitant decrease in CRE-luciferase activity, 70% of control activity was observed for the ratio 0.5/1.0, and 54% for 1.0/1.0 (µg/µg).

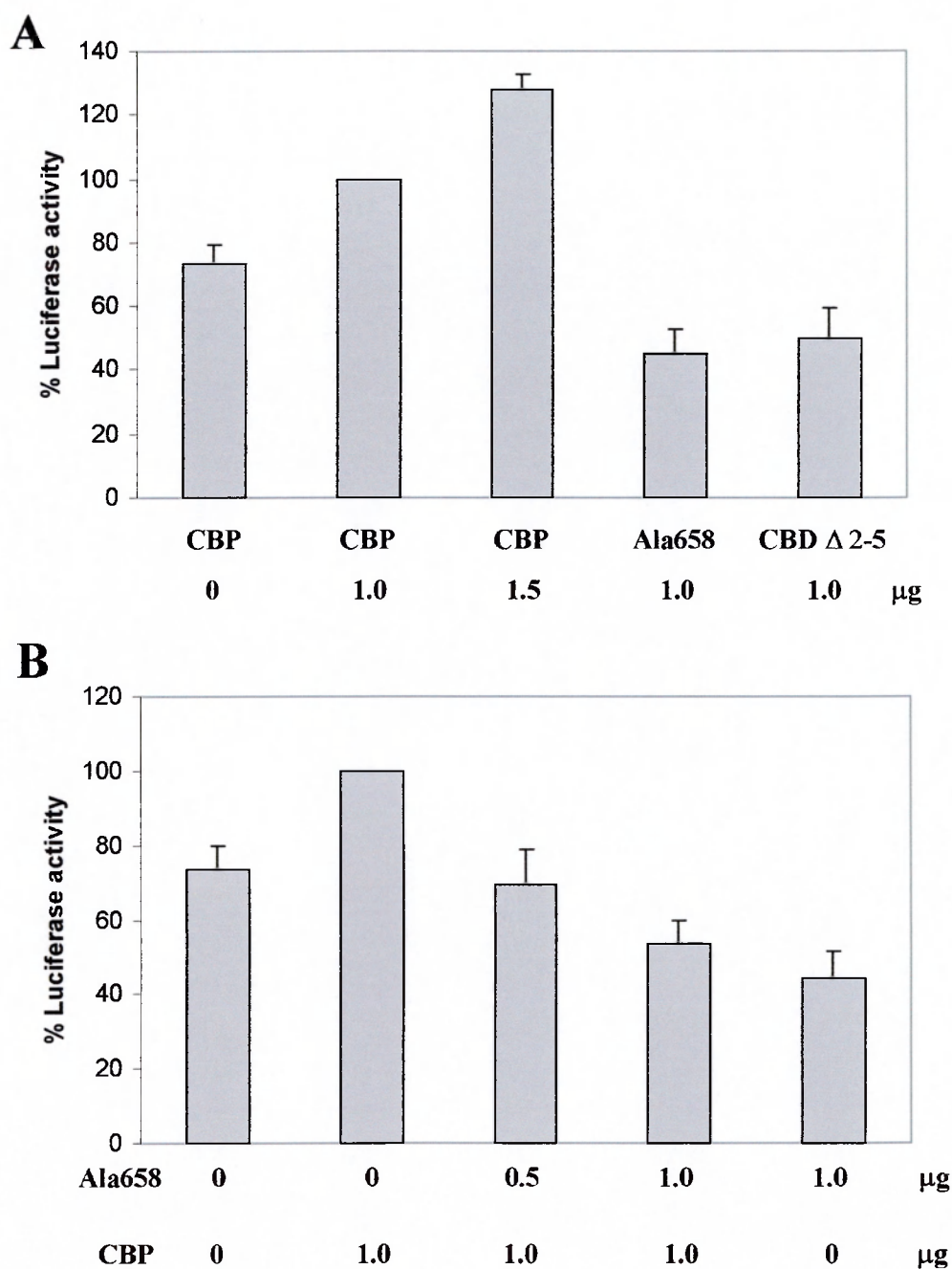


Fig. 3.10: CRE-luciferase activity induced by CBP wild-type and CBD mutants. **A.** Cotransfection of differentiated F9 cells with CBP wild-type (CBP) and mutants (CBPAla658 and CBPΔCBD2-5, respectively) together with pHD-CREB, pCEV (catalytic unit of PKA) and reporter construct CRE (CREB responsive element)-luciferase. **B.** Dose-dependent effect of CBPAla658 on cAMP pathway. Note that all data shown represent the means of three independent experiments, and that both CBPΔCBD2-5 and CBPAla658 have a dominant-negative effect on the cAMP signalling, and the effect of CBPAla658 was shown to be dose-dependent.

In order to investigate whether the mutations that affect the CREB-binding domain, and still retain other functions of CBP, for example, steroid hormone receptor-mediated transcriptional regulation, CBP wild-type and two CBP mutants (CBPA_{la658} and CBP Δ CBD2-5, respectively) were separately cotransfected into F9 cells together with the reporter construct GRE (glucocorticoid receptor responsive element)-luciferase. As expected, dexamethasone (Dex)-induced enhancement through GRE-luciferase was retained for both mutants CBPA_{la658} and CBP Δ CBD2-5 (Fig. 3.11). In comparison to transfection of wild-type CBP (normalized as 100%), 151% GRE-luciferase activity was observed with CBP Δ CBD2-5, and 142% with CBPA_{la658}. The fact that none of the mutants interfere with dexamethasone induction is consistent with that wild-type CBP being used as a transcriptional cofactor both by GR (glucocorticoid receptor) and by phospho-CREB, but the mutants being used as transcriptional cofactors only with GR. This could also be a reason for the fact that not much difference of GRE-luciferase activity was observed when comparing transfections with and without wild-type CBP (93% activity without CBP) (Fig. 3.11).

The RTS mutants were also evaluated for their functions in the cAMP signalling pathway. All three RTS mutants strongly inhibited expression of CRE-luciferase, in contrast to the wild-type CBP (Fig. 3.12). 81% luciferase activity was observed for the basal level (without CBP), only 36% activity for RTS1, 18% activity for RTS2 and 28% activity for RTS3. This indicated that RTS mutants might have dominant-negative effects on CBP function in the context of cAMP signalling. Other pathways have not yet been tested.

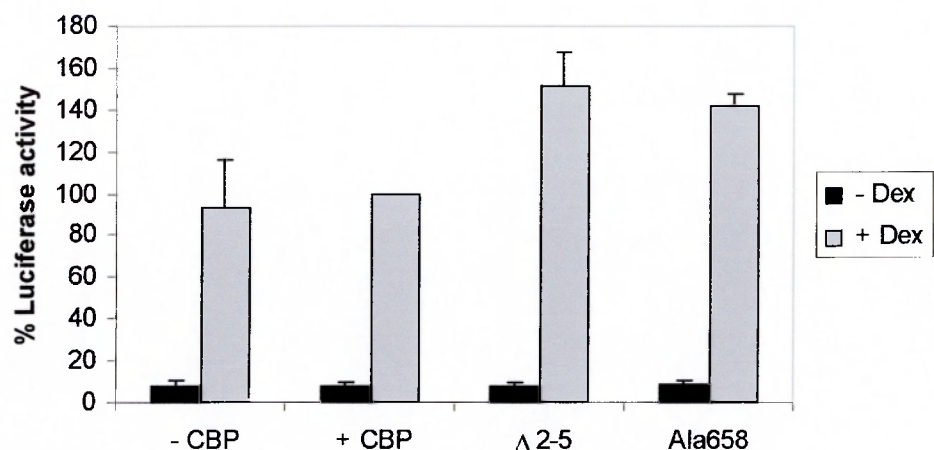


Fig. 3.11: GRE-luciferase activity induced by wild-type and mutant CBP, with (+Dex) and without dexamethasone (-Dex). In this experiment, the same amounts of wild-type and of each CBP mutant were applied in different transfections. Glucocorticoid-mediated transcriptional enhancement was retained in both CBPAla658 and CBPΔCBD2-5 mutants. All data shown represent the means of three independent experiments.

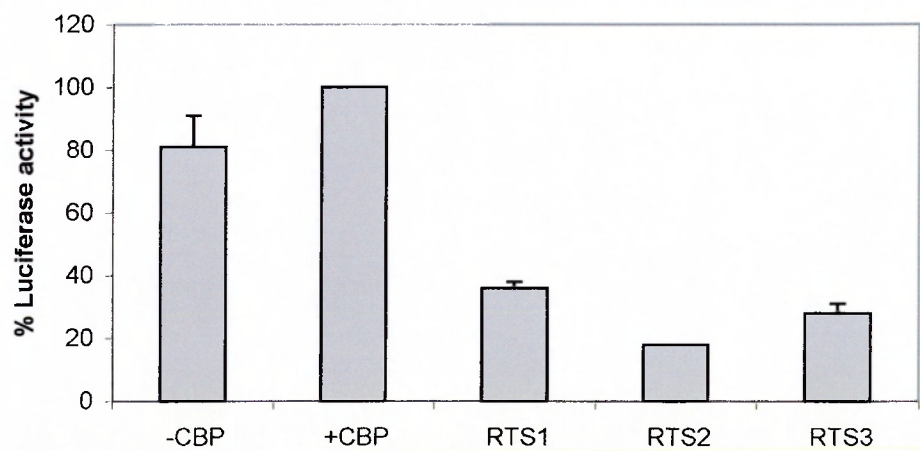


Fig. 3.12: CRE-luciferase activity induced by wild-type CBP and the RTS mutants. Cotransfection of differentiated F9 cells with CBP wild-type (CBP) and mutants (RTS1, RTS2 and RTS3, respectively) together with pHD-CREB, pCEV and reporter construct CRE - luciferase. Three RTS mutants displayed dominant-negative effect on CBP function in the context of cAMP signalling pathway. All data shown represent the means of three independent experiments.

3.3 Genomic organization of *CBP*

3.3.1 Isolation of genomic *CBP* DNA

To carry out gene targeting of the *CBP* gene, it was necessary to obtain mouse genomic clones of this gene. Genomic DNA fragments spanning the CREB-binding domain of *CBP* were isolated from λ phage genomic DNA libraries derived from embryonic stem cell lines E14 and D3. The libraries had been generated by insertion of *Sau3AI* partially digested fragments into λ DASHII phage vector digested with *Bam*HI, and were provided by Dr. Klaus Kaestner, Philadelphia, USA.

3.3.1.1 Preparation of probes for genomic DNA screening

Based on the *CBP* cDNA sequence available from GenBank (Accession number S66385), a 573-bp fragment from the transactivation domain (TAD) region (Swope *et al.*, 1996) and a 632-bp fragment from the CREB-binding domain (CBD) region (Chrivia *et al.*, 1993) of *CBP* were amplified by RT-PCR on RNA from adult mouse brain using two pairs of primers, Z1-Z2 and Z3-Z4 (see Table 2.6), respectively. The PCR products were subcloned into *Eco*RV-digested pBSII KS vector, and the subclones were designated as pBS-TAD.CBP and pBS-CBD.CBP. Both clones were sequenced and confirmed to be correct by comparison to the published *CBP* cDNA sequence. The hybridization probes were obtained by double digestion of the correct plasmids with *Hind*III and *Eco*RI, purified and used for hybridization after 32 P-labelling.

3.3.1.2 Cloning, restriction mapping and sequencing of *CBP*

After screening of the phage libraries, two independent phage clones containing part of genomic *CBP* were isolated. Following restriction mapping and Southern blot analysis, several genomic *CBP* restriction fragments were purified and subcloned into pBSII KS(-) plasmid vector. These smaller clones facilitated further analysis, including sequencing and construction of targeting vectors. All plasmid clones of genomic *CBP* were examined and confirmed by restriction enzyme digestion and sequencing. Sequencing was performed by MWG-Biotech (Ebersberg, Germany).

3.3.2 Genomic structure of *CBP*

3.3.2.1 Phage clones of genomic *CBP*

Several phages were identified which hybridized to the TAD and CBD probes and to oligonucleotide Z101, which is 30-nucleotides in length and is located at the 5' end of exon 5 of the CBD domain (referring to Table 2.6). Together, they comprised about 28 kb of genomic *CBP* sequence around the CREB-binding domain (CBD) (Fig. 3.13). Based on the digestion patterns and Southern blot analyses, two phage clones from the E14 library (E14-1 and E14-2) were positive for the CBD and the TAD region, while another three phage clones from the D3 library (D3-1, D3-2 and D3-3) were positive for the CBD region and the oligonucleotide Z101. This indicated that the TAD probe sequence is located furthest upstream in the region covered by the E14 phage clones in a region where the sequence is not yet known, that the CBD probe sequence is located on both phage clones, and that the D3 phage clones have more downstream sequences of *CBP*.

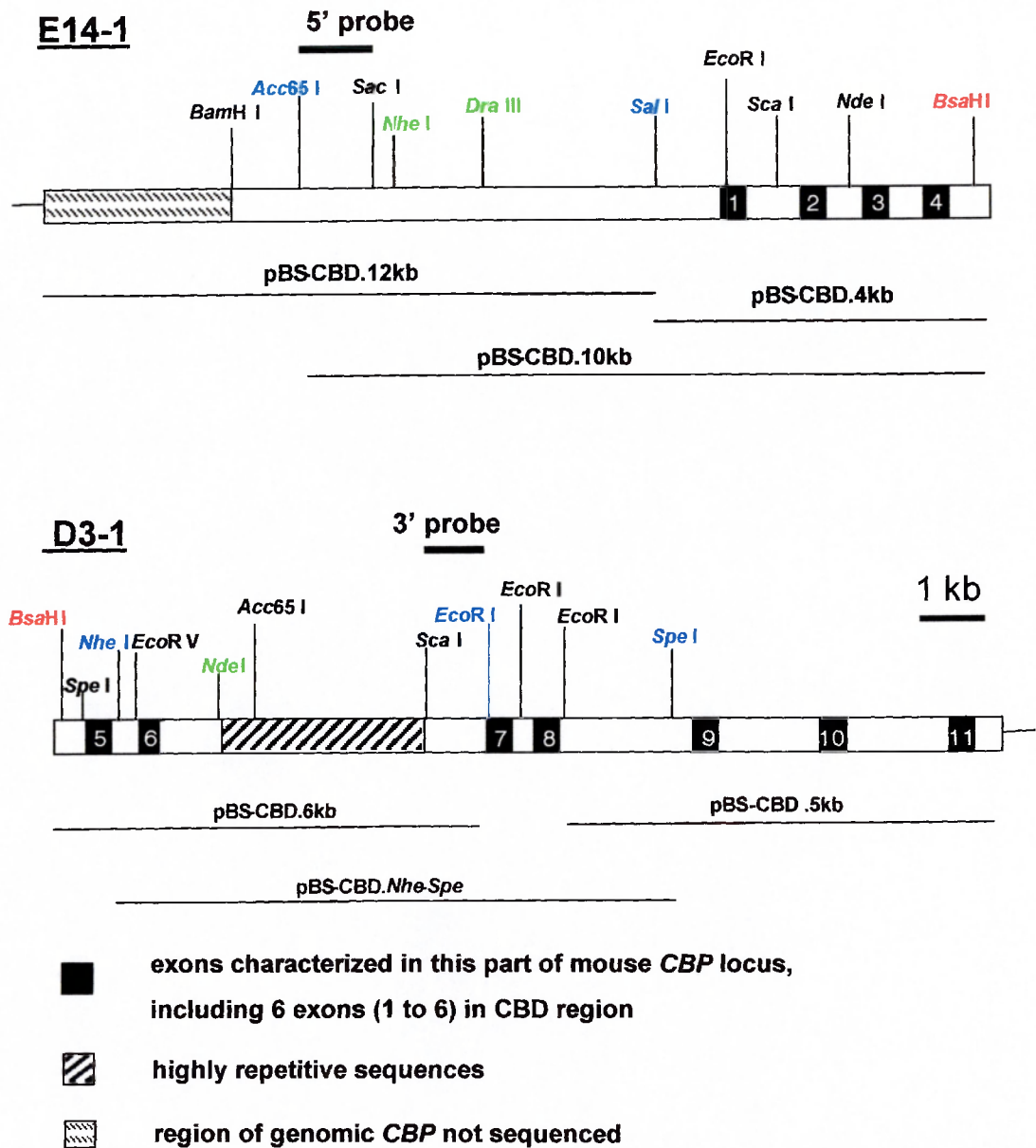


Fig. 3.13: Schematic representation of part of the mouse *CBP* locus around the CBD region. The two phage clones isolated comprise about 28 kb of genomic *CBP* sequence. The *Bsa*HI site used for ligation of the two phage inserts is marked in red. The restriction sites marked in blue were used for subcloning of parts of *CBP* into plasmid vectors, and sites marked in green were used for the ends of homology arms in the targeting constructs: *Nhe*I for the left arm of the targeting construct *CBP*^{stop523}, *Dra*III for the left arm of the targeting construct *CBP*^{Tyr658Ala}, and *Nde*I for the right arms of both constructs. 5' and 3' hybridization probes for the characterization of targeted ES clones are indicated with black bars. Important plasmid clones are indicated by black lines.

3.3.2.2 Plasmid clones of genomic *CBP*

I attempted to subclone the two inserts of genomic *CBP* into the pBSII KS plasmid vector, but only the insert from phage clone E14-1 was successfully subcloned. Firstly, the whole insert was isolated from an agarose gel after digestion with *Not* I, then subcloned into *Not* I-digested plasmid pBSII KS, which had been dephosphorylated with shrimp alkaline phosphatase (SAP). The correct plasmid clone was confirmed by restriction digestions and sequencing, and named pBS.E14.CBP.

Several subclonings were carried out for further evaluation of *CBP* genomic structure and subsequent construction of targeting vectors. The plasmid clones pBS-CBD.12kb, pBS-CBD.4kb and pBS-CBD.10kb were derived from the E14-1 phage clone (see Fig. 3.13). The plasmid clone of the whole E14-1 phage insert (pBS.E14.CBP) was digested by *Not*I and *Sal*I, then 12 kb *Not*I-*Sal*I and 4 kb *Sal*I-*Not*I fragments were isolated and subcloned into *Not*I and *Sal*I double digested pBSII KS vector. The 10 kb *Acc*65I-*Not*I fragment was also isolated from the pBS.E14.CBP clone and subcloned into the pBSII KS vector. The correct plasmid clones were confirmed by restriction digestions and sequencing.

A BLAST search using the isolated *CBP* genomic sequence (from the sequencing data in this thesis) as a query revealed about 2.3 kb of repetitive sequences (alignment score ≥ 200) at 1 kb downstream of CBD exon 5 in clone D3-1. This may have been the reason for the failure to subclone the whole phage insert into plasmid vector. I attempted unsuccessfully to subclone this fragment into four bacterial strains (DH5 α , XL-1 Blue, JM109 and SURE). The plasmids pBS-6kb, pBS-*Nhe*-*Spe* and pBS-5kb were therefore designed by subcloning 6 kb and 5 kb *Eco*RI fragments of the D3-1 clone, respectively, into pBSII KS (Fig. 3.13). Plasmid clones pBS-6kb and pBS-5kb were obtained, but

pBS-*Nhe-Spe* was very difficult to obtain in its full length: only subclones carrying truncated versions of this fragment were frequently obtained, possibly due to the repetitive sequence.

3.3.2.3 Structure of the *CBP* gene

About 23 kb of the 28 kb isolated genomic *CBP* were sequenced using convenient plasmid subclones. The sequence is depicted in Appendix II. There is only about 280 bp of sequence overlap between the two isolated phage clones E14-1 and D3-1, and the *Bsa*HI restriction site was the only possible site (Fig. 3.13) to combine the two phage inserts.

By comparing this genomic sequence with the cDNA sequence, the genomic structure of *CBP* was characterized and the exon/intron junctions of *CBP* in the CBD region were determined. Eleven exons, covering 2 kb of cDNA sequence, were found in the 28 kb isolated from the *CBP* locus. Of these, six exons were identified to be located in the CBD region (Fig. 3.13).

As the unique *Bsa*HI site was detected in both phage clones, E14-1 and D3-1, it was used to combine these two phage inserts. Several convenient restriction sites were identified for the construction of the targeting vectors (see Fig. 3.13).

Based on the sequencing data of exon/intron junctions in the CBD region, there are two possible ways to delete the CBD without disrupting the open reading frame of the downstream sequence of *CBP*. One is the deletion of CBD exon 3 and the other is a larger deletion of CBD exons 2 to 5. Deletion of exon 2 leads to a frameshift mutation, which introduces an in-frame stop codon just two amino acid residues downstream of

CBP residue 523, thus preventing expression of the region containing tyrosine 658, which is critical for CREB-binding activity and is encoded by CBD exon 5 (Fig. 3.14).

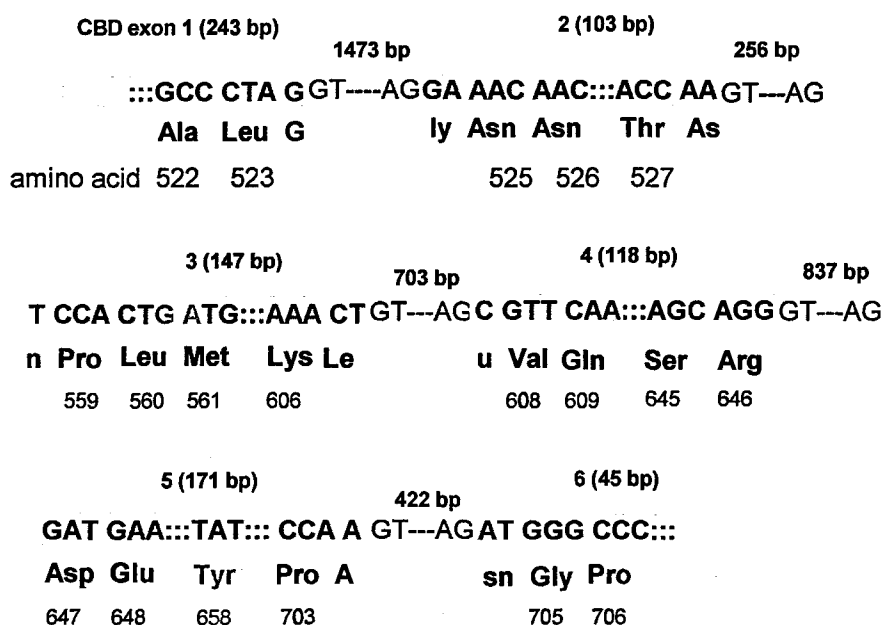


Fig. 3.14: Exon/intron structure of CREB-binding domain of *CBP*. There are six exons in this region. Deletion of CBD exon 3 or exon 2 to 5 will not disrupt the open read frame of the rest of *CBP*. Deletion of exon 2 leads to a frame shift mutation, and the new stop codon marked in pink will be introduced. The amino acid tyrosine 658 critical for binding of phosphorylated-CREB *in vitro* is encoded by exon 5.

3.3.3 Targeting constructs for generation of conditional *CBP* knock-out mice

In order to test the hypothesis mentioned in Chapter 1 that CBP might play an important role on the formation of LTM, it was proposed to inactivate essential exon(s) of the CREB-binding domain of *CBP* in a tissue-specific manner by using the Cre/loxP system. The knowledge of the genomic structure and restriction map of the CBD region of *CBP* allowed the design of appropriate targeting constructs to introduce relevant mutations into the mouse germline.

The first mutation is a deletion of CBD exon 2, which results in a frameshift of the rest of CBP and the introduction of a stop codon located at the third codon downstream of residue 523 (Fig. 3.15A). Thus, a truncated N-terminal CBP fragment of 523 amino acid residues together with two C-terminal amino acid residues unrelated to CBP is encoded by this lesion (named CBP^{stop523}). This mutation mimics the human genetic disease RTS (Petrij *et al.*, 1995). In the construct, CBD exon 2 was flanked by two loxP sites in a direct repeat orientation. The positive selection marker, phosphoglycerate kinase-neomycin phosphotransferase (PGK-Neo) was included between the two loxP sites and flanked by two FRT sites.

The second construct is a substitution of tyrosine to alanine at amino acid residue 658 in CBD exon 5 (CBP^{Tyr658Ala}), which should disrupt transcriptional enhancement through the cAMP pathway by abolishing binding of phospho-CREB to CBP (Radhakrishnan *et al.*, 1997). The point mutation construct (CBP^{Tyr658Ala}) was designed by using two mutant loxP sites (lox66 and lox71) in opposite orientations: the lox66 and lox71 sites flanked a sequence containing both the wild-type exon 5 and an inverted copy of exon 5 with the Tyr658Ala substitution (Fig. 3.15B). The PGK-Neo cassette was introduced between two mutant loxP sites as positive selection marker. The thymidine kinase (tk) cassette was placed at the end of the right homology arm as negative selection marker, because of the repetitive sequences downstream of CBD exon 6. And to avoid including these repetitive sequences in the targeting construct, only 1 kb of sequence is available for the right homology arm. The DNA sequence flanked by the mutant loxP sites was expected to be almost fully inverted and the mutated exon 5 was then put into a position to be transcribed and correctly spliced when the floxed mouse line was crossed with Cre recombinase-expressing mice.

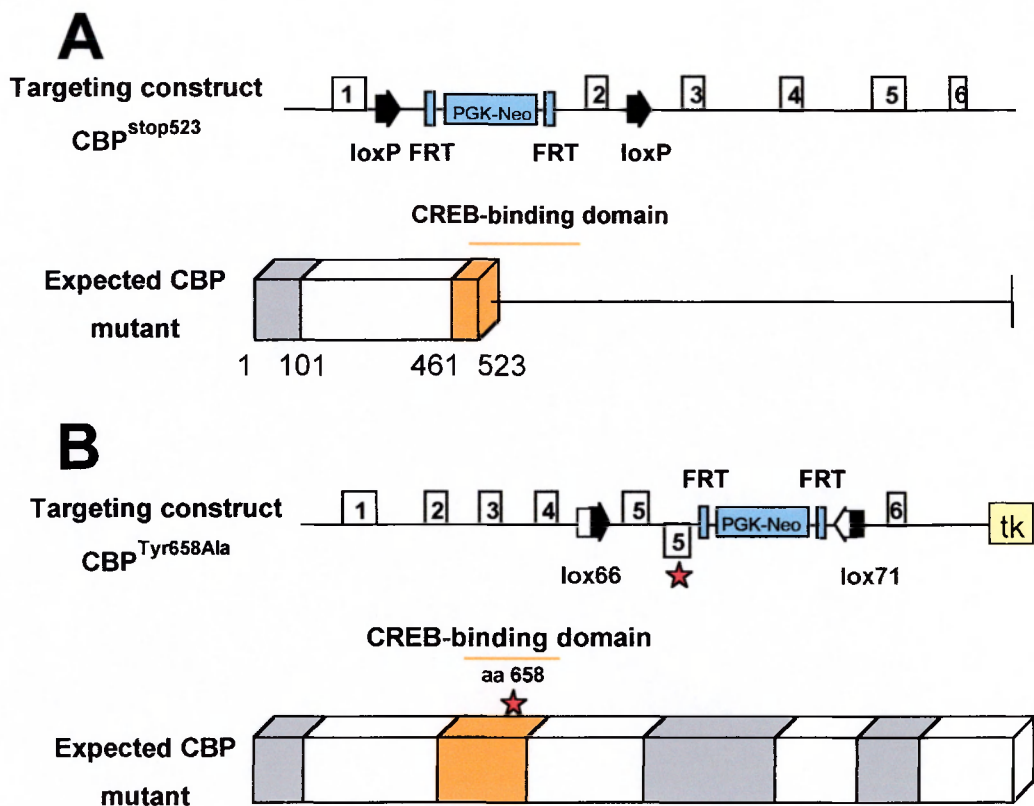


Fig. 3.15: Schematic representation of the two targeting constructs and their expected protein products after Cre-mediated recombination. PGK-Neo cassette flanked by two FRT sites are marked in pale blue; tk cassette is marked in yellow; CBD is marked in orange and aa 658 is indicated by red asterisk; Other domains of CBP are marked in grey and white (for detail, see Fig. 1.5). A. Deletion mutant CBP^{stop523}. B. Point mutant CBP^{Tyr658Ala}

3.4 Probes for screening for correct homologous recombinants

As described below in sections 3.5.1 and 3.6.1, both constructs share the same probes for diagnosis of correct homologous recombination.

3.4.1 Preparation of probes

5' probe. The clone pBS-CBD.12kb was digested with *Acc65I* and *SacI*, then the 1.4 kb fragment (Fig. 3.13) was isolated using agarose gel electrophoresis and purified using

a QIAGEN gel extraction kit. Both sticky ends of the fragment were made blunt using T4 polymerase. The 1.4 kb fragment was then subcloned into *Sma*I-digested pBSII KS to give pBS-5'-probe. The 5' probe was obtained by double digestion of pBS-5'-probe with *Bam*HI and *Pst*I followed by purification of the 1.4 kb fragment.

3' probe. The clone pBS-CBD.6kb was digested with *Sca*I and *Eco*RI, and the 820 bp fragment (Fig. 3.13) was isolated and purified. The *Eco*RI end of the fragment was made blunt using T4 polymerase. The 820 bp fragment was then subcloned into *Sma*I-digested pBSII KS to give pBS-3'-probe. The 3' probe was obtained by double digestion of pBS-3'-probe with *Bam*HI and *Pst*I followed by purification of the 820 bp fragment.

Neo probe. The clone pBS-PGK.Neo (obtained during the cloning steps of the targeting constructs) was digested with *Xba*I. A 300 bp fragment from the *neo* gene (see Fig. 3.26 and 3.43) was then purified and subcloned into *Xba*I-digested pBSII KS to give the plasmid pBS-Neo-probe. The Neo probe was obtained by double digestion of pBS-Neo-probe with *Not*I and *Pst*I followed by purification of the 300 bp fragment.

3.4.2 Qualitative analysis of probes

Reverse Southern blotting was used to test whether the probes used contained repetitive sequence. Phage clones E14-1 and D3-1 were digested with different restriction enzymes, including the digestions used for generation of the 5' and 3' probes. Blots of these digests were hybridized with ³²P-labelled genomic DNA from the 129/Ola mouse strain. Control DNAs (clones p12/3 SN6000 and p12/3 NS9000, from the orphan nuclear receptor Ear2 gene, obtained from Dr. Beat Lutz) were included in the analysis, as it was known that these clones contain some repetitive sequences.

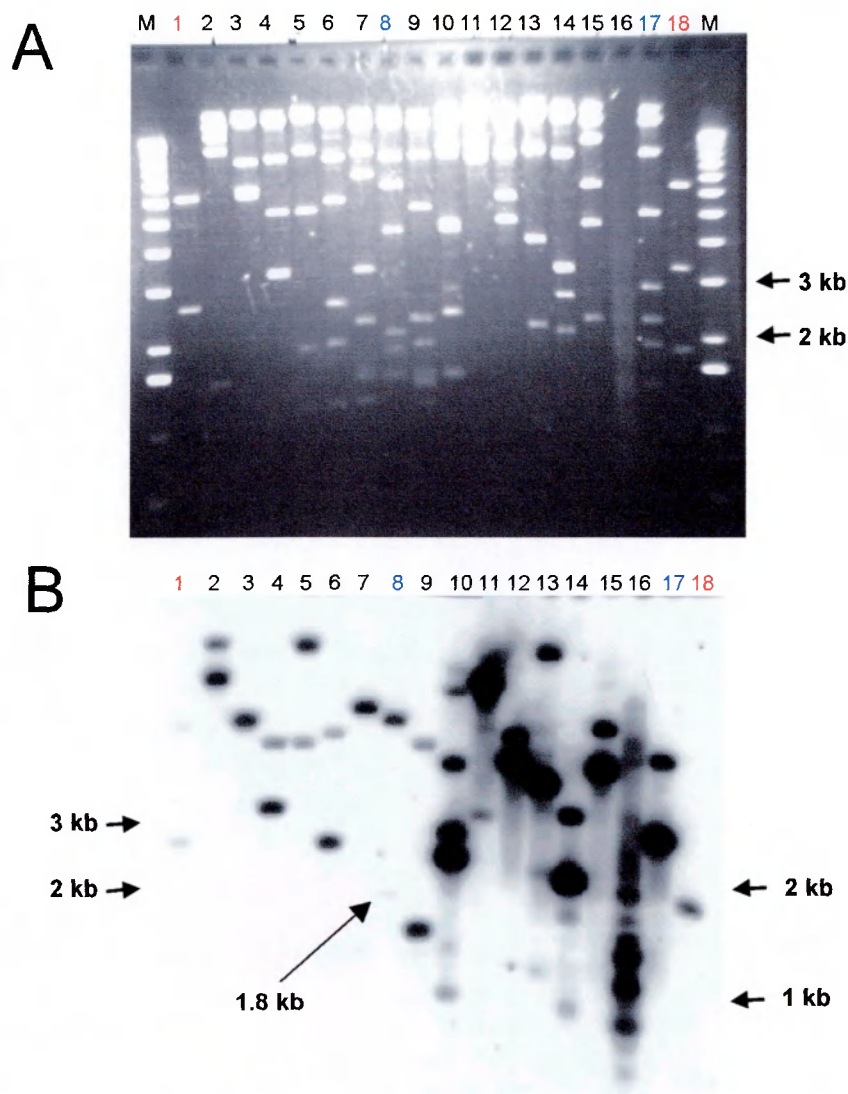


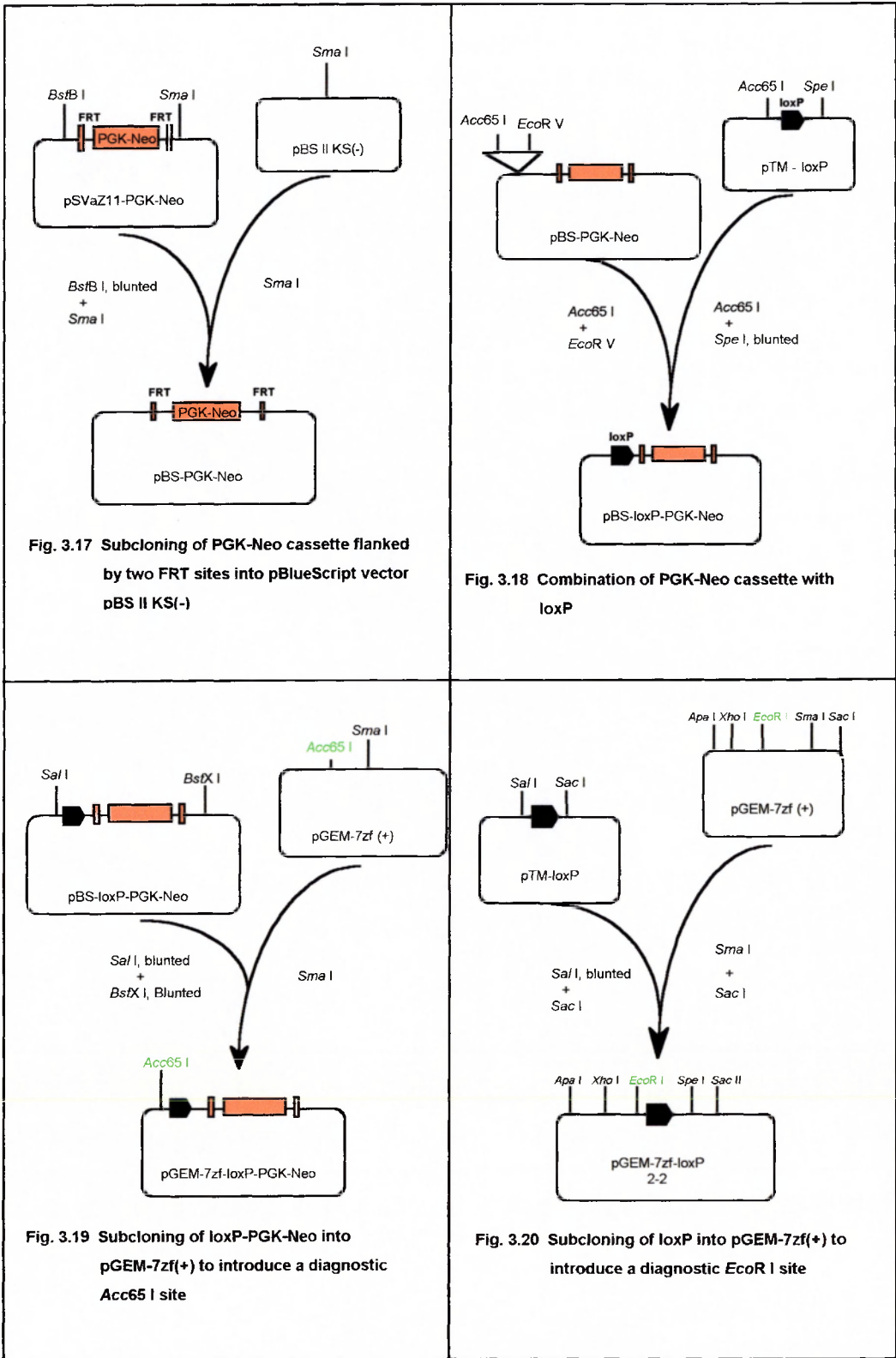
Fig. 3.16: Qualitative analysis of the diagnostic probes using a reverse Southern blot. **A.** Agarose gel electrophoresis of different digestions of CBP phage clones. **B.** Reverse Southern blot pattern obtained with ^{32}P -labelled genomic 129/Ola DNA. Lane 1 and 18 are marked in red and are the two control DNAs, SN6000/*Xba*I and NS9000/*Eco*RI, respectively; lanes 2-17 are different digestions of the two CBP phage clones. Among them, lanes 8 and 17 marked in blue are *Acc*65I-*Nhe*I digestion of phage E14-1 and *Eco*RI-*Sca*I digestion of phage D3-1, which contain the 5' probe and 3' probe, respectively. Note: lane 8 showed a positive band of 1.8 kb, indicating repetitive sequences. No positive band of 0.8 kb was found in lane 17, therefore, the 3' probe is good for Southern blot screening. Refer to Fig. 3.13 for a restriction map that shows the sites cut by the relevant restriction enzymes.

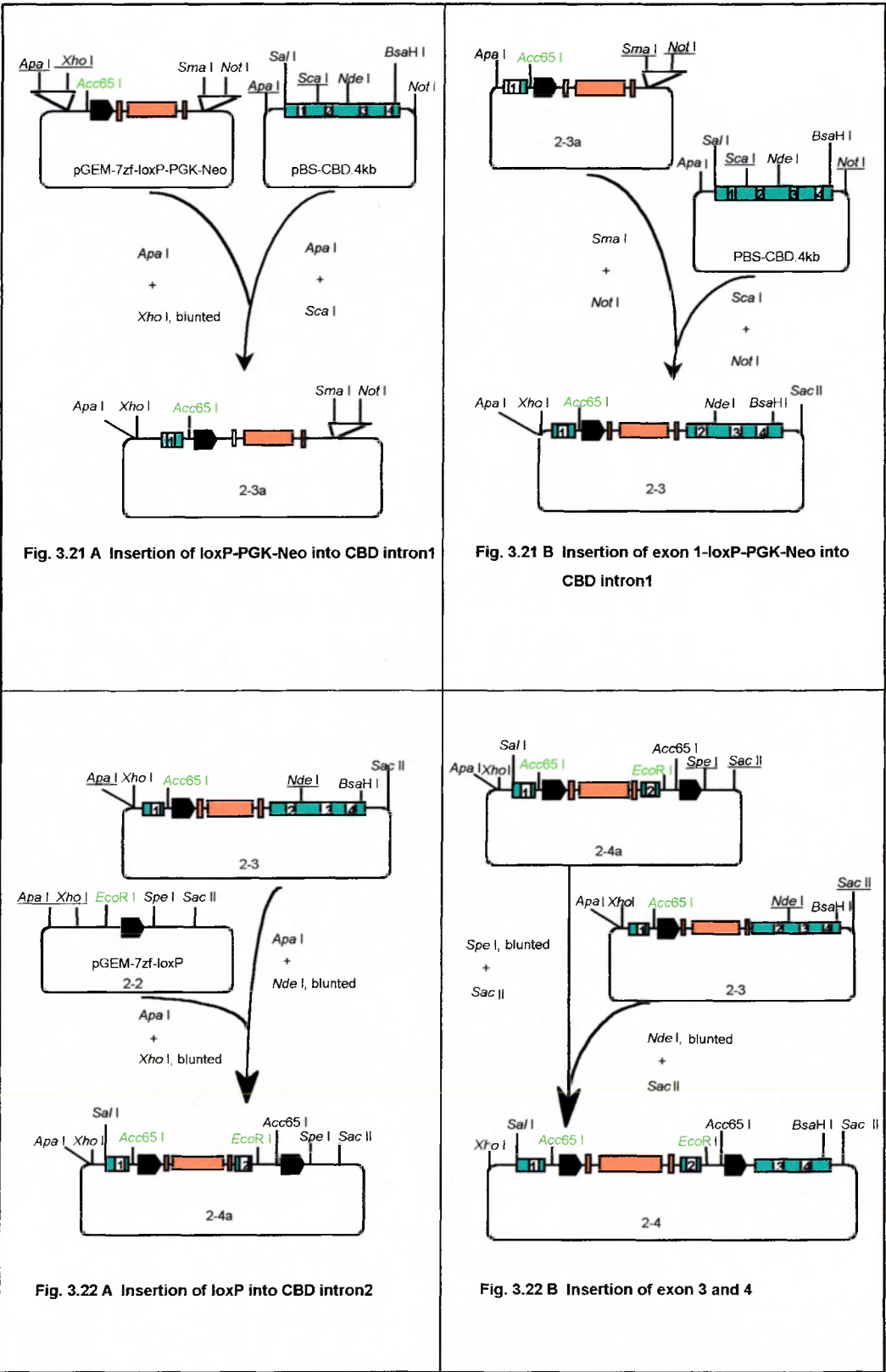
A reverse Southern blot with ^{32}P -labelled mouse strain 129/Ola genomic DNA showed that the two control DNA samples showed hybridization signals as expected, that the 3' probe is good for Southern blot screening, while the 1.8 kb *Acc65I-NheI* fragment contains repetitive sequences (Fig. 3.16). The 5' probe was obtained by *Acc65I* and *SacI* double digestion of pBS-CBD.12kb, excluding a 0.4 kb *SacI-NheI* fragment (referring to Fig. 3.13). This 1.4 kb fragment was confirmed to be devoid of repetitive sequences by a reverse Southern blot and a BLAST search (data not shown).

3.5 Generation of $\text{CBP}^{\text{stop523}}$ knock-out mouse

3.5.1 Construction of the targeting vector $\text{CBP}^{\text{stop523}}$

To generate a mouse model for the human genetic disease RTS, targeting vector $\text{CBP}^{\text{stop523}}$ (Fig. 3.17 to 25) was constructed, in which CBD exon 2 was flanked by two loxP sites, leading to deletion of whole CBD exon 2 and a frameshift, and introduction of a new stop codon. A truncated protein with only N-terminal 523 amino acids of CBP is encoded by this mutation. To achieve this, two loxP sites in the same orientation were introduced 5' and 3' of exon 2 in the targeting vector. A PGK-Neo cassette was also introduced as a positive selection marker. ES cells containing this antibiotic resistance gene have the ability to survive in the presence of the neomycin derivative geneticin (G418). The integrity of the final targeting vector was confirmed by sequencing of all critical regions, i.e. loxP sites, FRT sites, exons and cloning junctions.





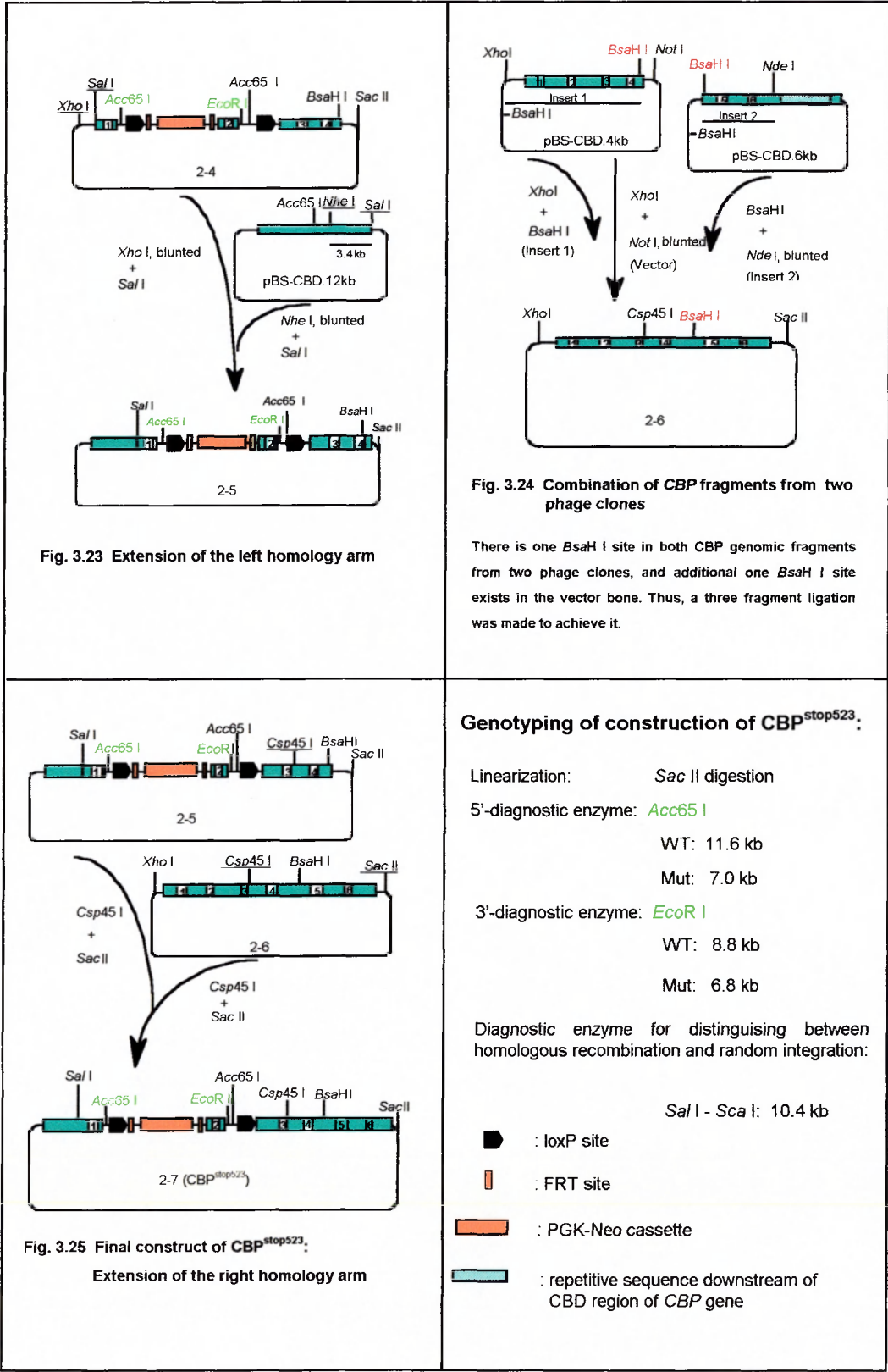


Fig. 3.17 to 25: Flow chart of construction of the *CBP*^{stop523} targeting construct. CBD exon 2 is flanked by two loxP sites in the same orientation. The PGK-Neo cassette, flanked by two FRT sites in the same oriaitaon, is included between the two loxP sites as a positive selection marker.

3.5.2 Screening for the homologous recombinants of the targeted $CBP^{stop523}$ allele

100 μ g of the targeting construct $CBP^{stop523}$ (Fig. 3.25) was fully linearized by *Sac* II digestion and confirmed by agarose gel electrophoresis. 20 μ g of the linearized clone was electroporated into mouse E14 ES cells. After nine days of G418 selection (200 μ g/ml), resistant colonies were visible. 300 single colonies were picked into 96-well feeder plates, and then expanded on 96-well feeder plates for freezing and on 96-well gelatinized plates for Southern blot analysis. The screening strategy is shown in Fig. 3.26.

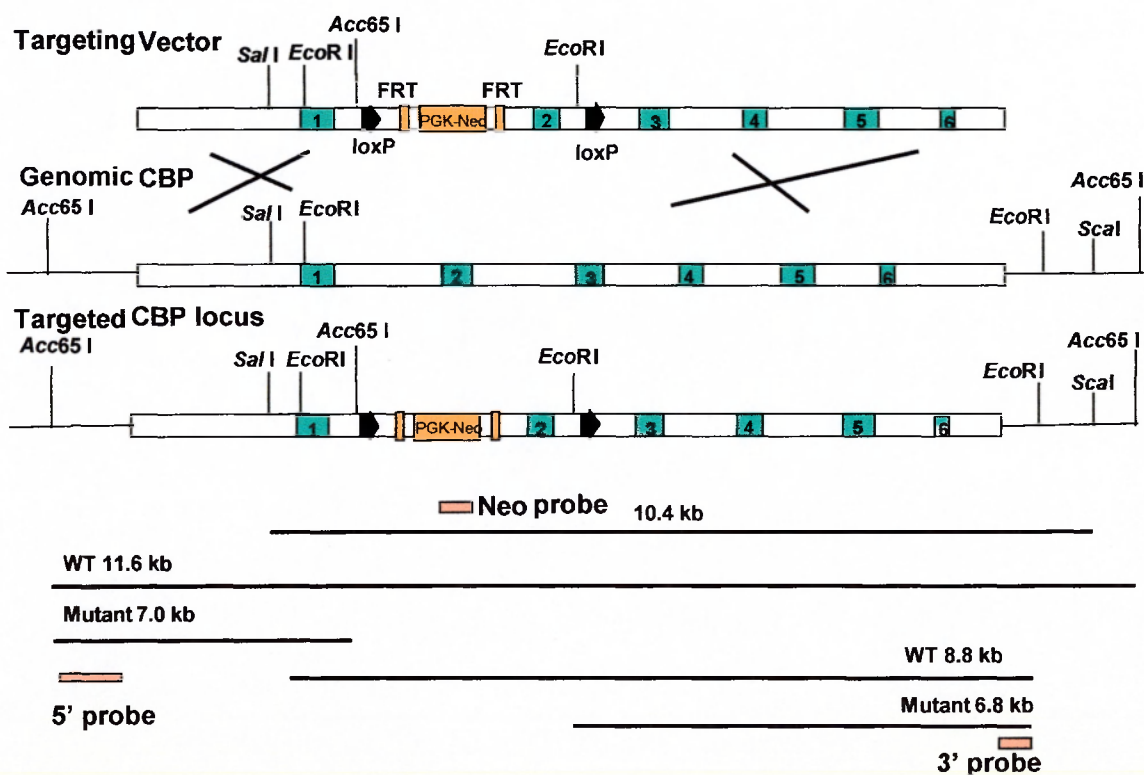


Fig. 3.26: Schematic representation of homologous recombination between the targeting construct $CBP^{stop523}$ and CBP gene locus. Diagnostic patterns of restriction fragments are also indicated: the 5' probe with *Acc*65I digestion was used for screening for correct homologous recombination of the left arm, the 3' probe with *Eco*RI digestion for correct homologous recombination of the right arm, and the Neo probe for exclusion of random integration events. Note: Fragment sizes are not drawn to scale.

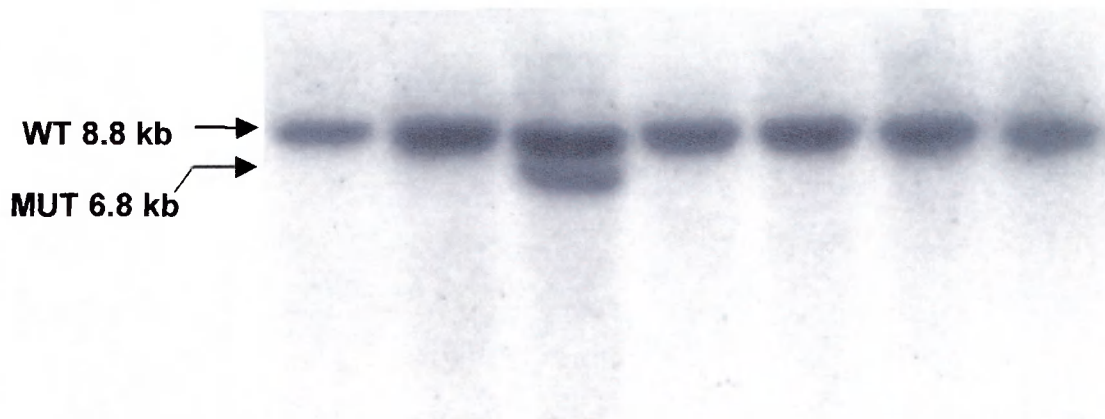
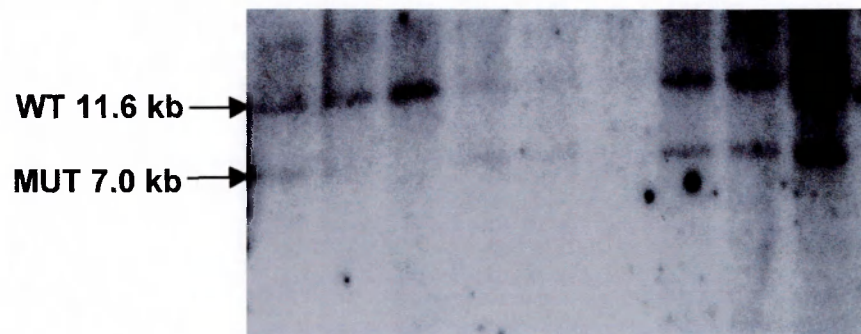
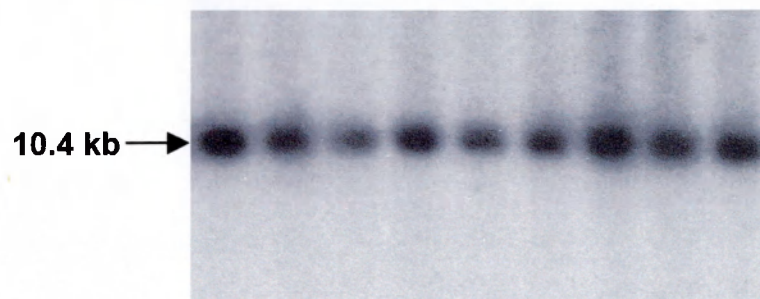
A. 3' probe screening**B. 5' probe screening****C. Neo probe screening**

Fig. 3.27: Screening for correct recombinants of the targeting construct $CBP^{stop523}$ using Southern blot analysis. A. Screening for the correct right arm with the 3' probe. Digestion with *EcoRI* gives an 8.8 kb band for the wild-type allele and a 6.8 kb band for the mutant allele. B. Screening for the correct left arm with the 5' probe. Digestion with *Acc65I* gives an 11.6 kb band for the wild-type allele and a 7.0 kb band for the mutant allele. C. Screening of 9 clones with the mutated 5' and 3' fragments for non-random homologous recombination with the Neo probe. Digestion with *SalI* and *ScaI* shows one single band of 10.4 kb, and no additional bands were visible. Note: A, B and C are not duplicate blots, but contain DNA from different subsets of G418-resistant cell lines.

As the PGK-Neo selection cassette was inserted into CBD intron 1, and close to the left homology arm, it is more likely that G418-resistant ES cell clones, whether or not they arose by homologous recombination, will include the left homology arm. Thus, the 3' probe for diagnosis was first used for the primary Southern blot screening with *EcoRI*-digested genomic DNA. The strategy and results of screening for the homologous recombinants of CBP^{stop523} are shown in Fig. 3.27.

Southern blot analysis showed that 9 of 300 (recombination efficiency: 3%) picked clones gave the expected pattern of one 8.8 kb wild-type band and one 6.8 kb targeted band. A further Southern blot analysis of *Acc65I* digestions using the 5' probe revealed that all these 9 positive clones showed one wild-type 11.6 kb and one targeted 7.0 kb band (see Fig. 3.26 for predicted restriction map). These 9 positive clones were further subjected to Southern blot analysis to examine possible random integrations, but all showed one single band of the expected size of 10.4 kb after *SalI* and *ScaI* double digestions and hybridization with Neo probe. Therefore, these 9 ES cell clones were good candidates to be injected into blastocysts for the germline.

3.5.3 Mating for germline transmission

E14 cells were originally derived from the 129/Ola mouse strain. Therefore, injection of these cells into C57BL/6 blastocysts will yield chimeras which can manifest coat color chimerism. The alleles of coat color genes that are present in 129/Ola are as follows: *Agouti*, *A^w* which is paler than A and encodes agouti color; *Albino*, *C^{ch}* (Chinchilla) which displays a reduction of tyrosinase activity and a reduction of deposition of yellow and, to a lesser extent, black pigment. The alleles carried by C57BL/6 are: *Agouti*, *a* which displays plain black pigment; *Albino*, *+^c*. Thus, chimeras contain a

creamy/brownish color on black. Mating of the chimeras with C57BL/6 mice results in E14-derived agouti colored pups and C57BL/6-derived black pups. As all the ES cell lines are male, germline transmission is usually through male chimeras.

Four E14 cell clones (SA6, S3A3, M2C4 and M2D6) positive for the targeted $CBP^{stop523}$ allele were selected for injection into C57BL/6 blastocysts. The percentage of E14-derived coat was evaluated based on coat colour of the chimeras. The result of blastocyst injection of the targeted $CBP^{stop523}$ cell lines is summarized in Table 3.2.

Table 3.2 Summary of blastocyst injections of the targeted $CBP^{stop523}$ ES cells

Cell clone	Number of			
	injected embryos	Number of born pups	Number of chimeras (% of E14-derived coat)	Germline transmission
SA6	197	17	3 (10, 20, 20%)	No
S3A3	107	4	1 (50%)	No
M2C4	212	22	5 (10, 40 , 50, 80, 100%)	Yes
M2D6	126	9	2 (50, 60%)	No

Eleven mice (6 males and 5 females) with 10-100% E14-derived coats were born, and all were mated at the age of 8 weeks with wild-type C57BL/6 mice to check for germline transmission. Only one male with 40% of its coat derived from E14, from cell line M2C4, gave agouti pups. About 1 cm of tail was cut from 3 week-old offspring of chimeras for genotyping. Germline transmission of the targeted allele was examined by PCR of the tail DNA with the primers Z37 and Z38 (referring to Table 2.6). Six of 9 agouti pups were confirmed to contain the mutated *CBP* allele (Fig. 3.28A, B). Forward primer Z37 is located in CBD exon 2 and reverse primer Z38 at the end of CBD intron 2.

A 230 bp fragment was amplified from the wild-type allele. Because of the insertion of loxP, a 300 bp fragment was amplified in the mutated *CBP* allele. Both bands were easily distinguishable on a 2.0% agarose gel (Fig. 3.28C). A single lower 230 bp band indicates mice homozygous for wild-type *CBP*, two bands (230 and 300 bp) indicate a heterozygous *CBP*^{stop523} genotype, and a single 300 bp upper band indicates homozygosity for the *CBP*^{stop523} allele.

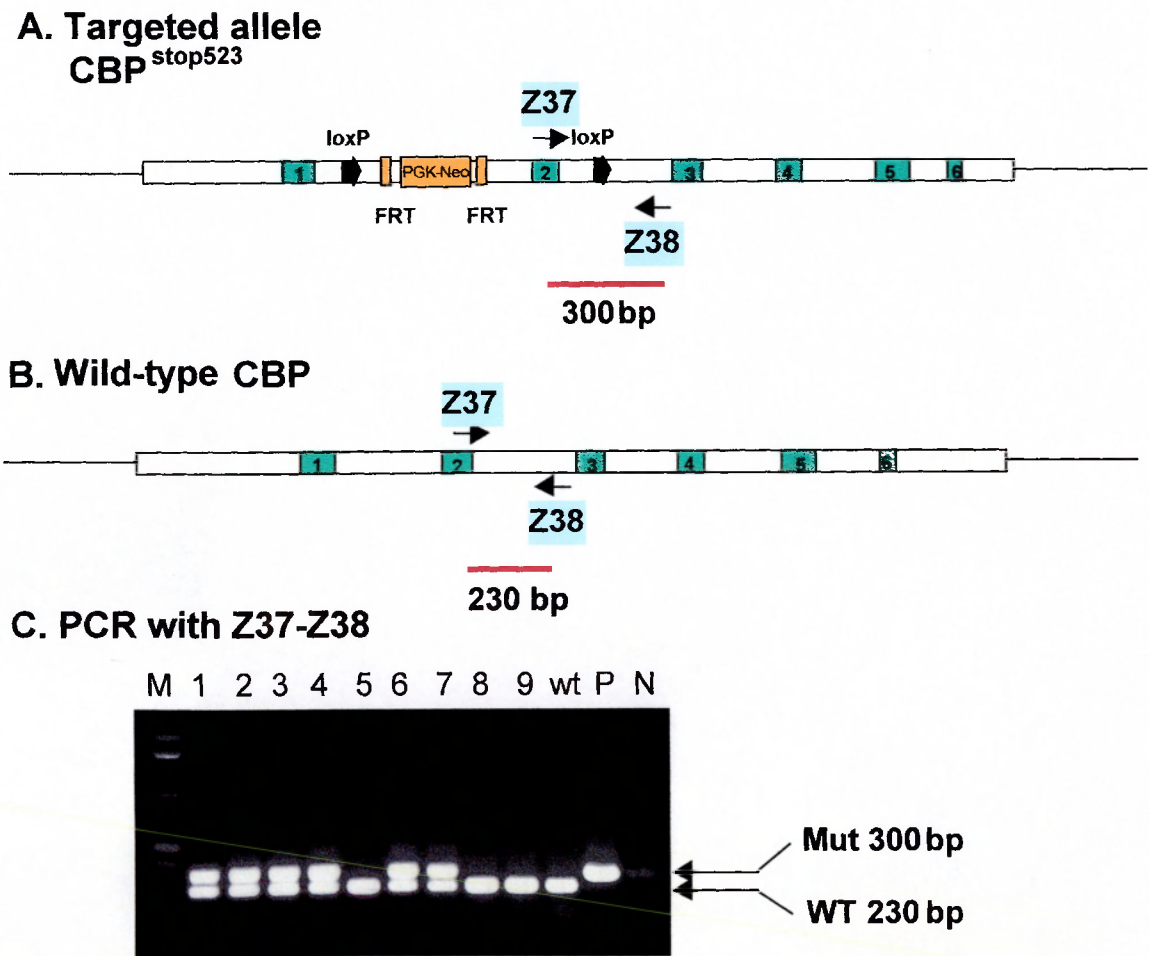


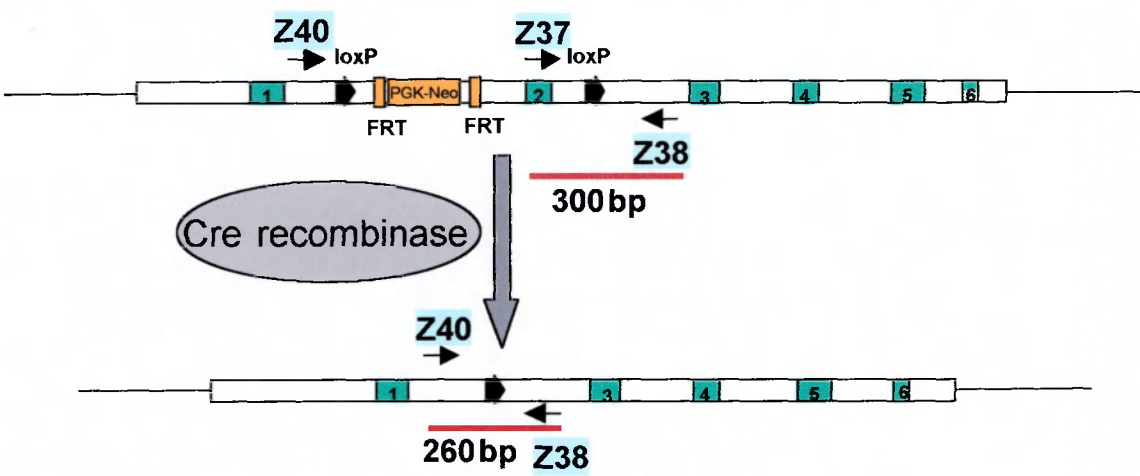
Fig. 3.28: Examination of the germline transmission of the targeted *CBP*^{stop523} allele by PCR. A. Targeted *CBP*^{stop523} allele. B. Wild-type *CBP* allele. C. Testing for germline transmission by PCR using primers Z37 and Z38, six of 9 agouti samples (1 to 9) show a 230 bp band for the wild-type allele and a 300 bp band for targeted allele. M: DNA marker, wt: wild-type mouse genomic DNA, P: the final plasmid clone of the targeting construct as positive control, N: H₂O as negative control.

3.5.4 Generation of a mouse model for human RTS

Crossing with Cre deleter mice. Three heterozygous males with the targeted $CBP^{stop523}$ allele were crossed with a Cre deleter mouse line (Schwenk *et al.*, 1995), in which Cre recombinase is expressed under the control of the human cytomegalovirus (CMV) minimal promoter sequence (P_{Bi-2}) (Baron *et al.*, 1995). The Cre deleter mice highly express Cre recombinase in almost all types of tissues, including germ cells. The Cre-mediated excision was checked by PCR of tail DNA using primers Z40 and Z38. A control PCR was also included using primers Z37 and Z38 (Table 2.6; Fig. 3.29A,B). The forward primer Z40 is located in CBD intron 1 and the reverse primer Z38 in intron 2. These primers amplified a 750 bp fragment from wild-type mouse genomic *CBP*, and a 260 bp fragment from the targeted allele in which CBD exon 2 and PGK-Neo cassette were excised by Cre recombinase. In principle, a 2.6 kb band from the targeted allele which contains CBD exon 2 and PGK-Neo cassette should also be amplified if Cre-mediated excision did not occur. However, this fragment was too big to be amplified simultaneously with the other fragments (Fig. 3.29C).

The genotyping of the progeny of this cross was performed by PCR using primers Z40 and Z38. Only 1 of 24 pups from 3 litters was positive for the Cre deletion. The DNA from this mouse (#62) was used as template for another PCR using primers Z37 and Z38 to confirm the excision (Fig. 3.29D). Because of the mosaic situation of deleted and non-deleted CBD exon 2, F1 offspring still contain the 300 bp band of the targeted allele. The positive F1 offspring (#62) was further mated with C57BL/6 mice to obtain non-mosaic mice heterozygous for the Cre-mediated deletion.

A. Cre-mediated recombination of targeted $CBP^{stop523}$ allele



B. Wild-type CBP

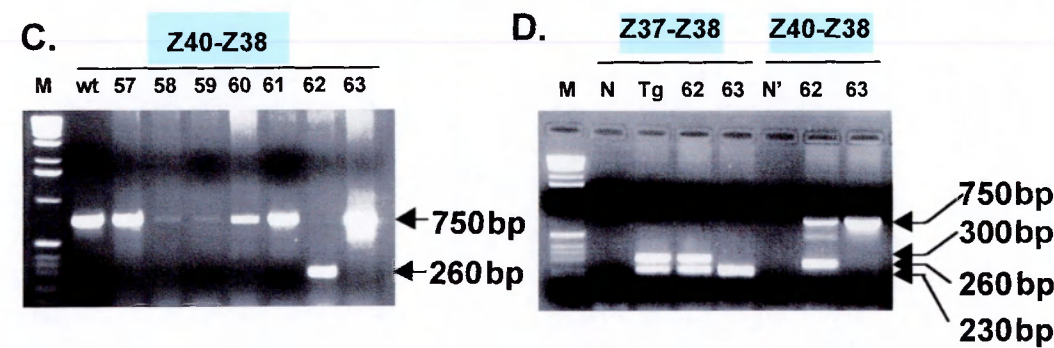
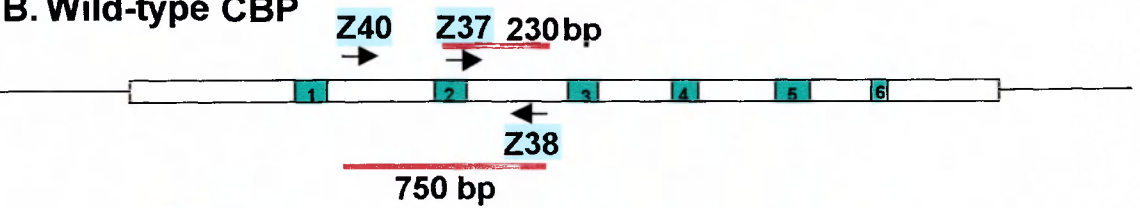


Fig. 3.29: Examination of Cre-mediated excision in the targeted $CBP^{stop523}$ allele by PCR. A. Targeted $CBP^{stop523}$ allele in which CBD exon 2 and PGK-Neo cassette are excised by Cre recombinase. B. Wild-type CBP allele. C. Testing for Cre-mediated deletion by PCR with primers Z40 and Z38. The 260 bp band results from deletion of CBD exon 2 (mouse #62) and the 750 bp band from wild-type genomic CBP (the others). D. The control PCR with primers Z37 and Z38 shows the 300 bp band for the targeted allele, and the 230 bp band from wild-type genomic CBP . M: DNA marker. N and N': H₂O as negative controls for the two pairs of primers used. wt: tail DNA from wild-type mouse. Tg: tail DNA from the targeted $CBP^{stop523}$ mouse. Numbers 57 to 63: tail DNA from the F1 offspring from the mating of the targeted mice with Cre deleter mice.

Two of six F2 offspring examined were shown to be heterozygous for the CBD exon 2 deletion, using PCR with primers Z40 and Z38. Control PCR with primers Z37 and Z38 indicated that mice #291 and #292 are non-mosaic heterozygotes (Fig. 3.30), as both mice show only the 230 bp band of the wild-type allele. This allele was named CBD2. Meanwhile, the presence of Cre recombinase was also examined by PCR using primers B42 and B43 which gave about 600 bp fragment for Cre recombinase (data not shown).

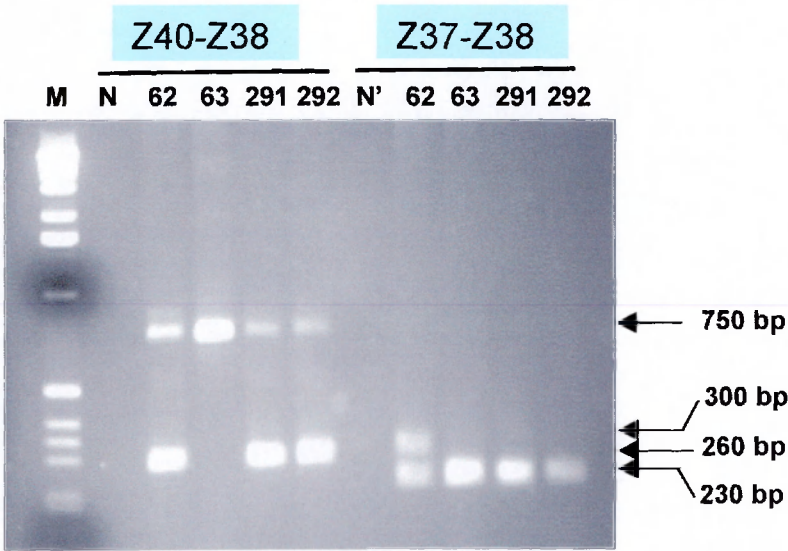


Fig. 3.30: Genotyping of F2 mice obtained after Cre-mediated deletion. Primers Z40-Z38 were used to check for Cre-mediated deletion, Z37-Z38 were used to check both for the presence of the 3' loxP site and the wild-type CBP allele (Fig. 3.29). M: DNA marker, N and N': negative controls for the two pairs of primers used, Numbers 62, 63: Tail DNA from F1 offspring of Cre-mediated excision. Numbers 291, 292: Tail DNA from F2 mice that were progeny of F1 mouse #62. Note that the F1 mouse #62, positive for Cre-mediated deletion, is mosaic, as control PCR using primers Z37-Z38 still shows the 300 bp band from the targeted allele. While the F2 offspring (named CBD2) are non-mosaic heterozygous mice, as the control PCR only shows the 230 bp band from the wild-type allele.

3.5.5 Generation of mice lacking the Neo cassette

In addition to generating mice carrying the CBD2 allele, which should express CBP^{stop523} in all tissues, I wished to generate mice in which tissue-specific Cre expression could be used to express this product only in specific tissues. However, the Neo selection marker gene should be removed from the targeted allele before mating with different Cre-expressing mouse lines to generate conditional *CBP* knock-out mice, since the Neo cassette inserted into an intron of the *CBP* gene might influence its expression. Several mice heterozygous for the targeted CBP^{stop523} allele were crossed with “Flp-deleter” mice in which Flp recombinase is ubiquitously expressed under the control of the human β -actin gene (*hACTB*) (Dymecki, 1996). Because of the rather low efficiency of Flp-mediated recombination, F1 mice are likely to be mosaic. Therefore, these mice were mated with C57BL/6 mice to allow screening for non-mosaic heterozygous F2 mice, named CBD2.floxed mice. Removal of the Neo cassette was determined by PCR of tail DNA with primers Z22 and Z39 (Table 2.6; Fig. 3.31). Firstly, mice that still retained the Neo gene were identified as being negative in this PCR reaction. Crossings between these mice were performed to generate mice homozygous for the construct that still retained the Neo gene (CBD2.floxed.neo), which can be used to analyse possible mutagenic effects of the Neo cassette (Artelt *et al.*, 1991; Fiering *et al.*, 1993; Rijli *et al.*, 1994) on *CBP* expression. Secondly, sixteen of 115 F1 generations were detected as positive for Neo deletion by PCR, showing a fragment 220 bp for the deletion using the same primers (Fig. 3.31).

A. Flp-mediated recombination in targeted $CBP^{stop523}$

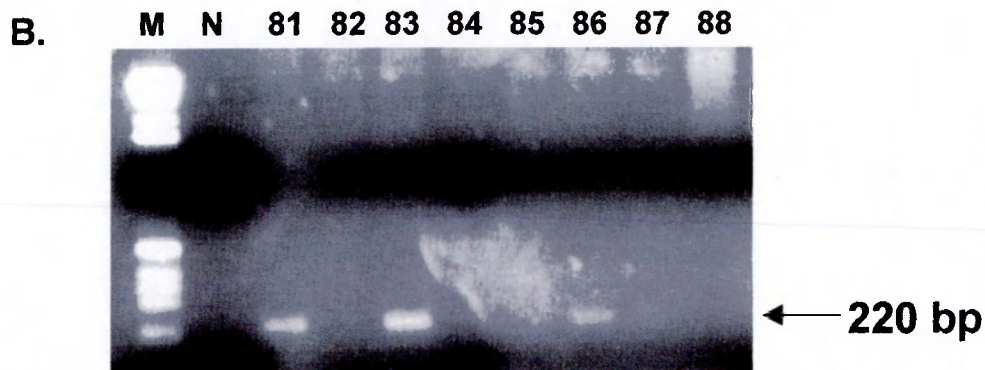
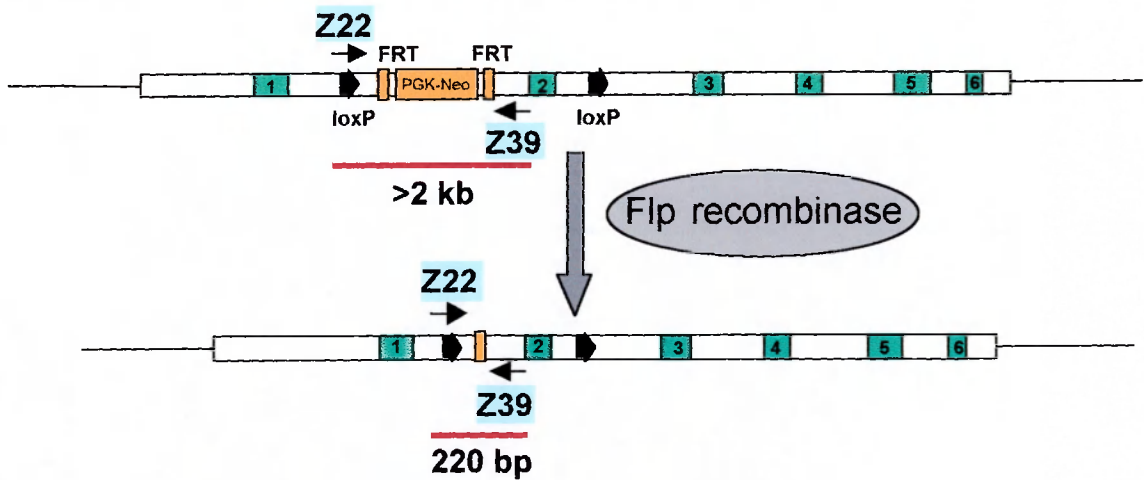


Fig. 3.31: Examination of FLP-mediated excision in the targeted $CBP^{stop523}$ allele by PCR with primers Z22 and Z39. **A.** Targeted $CBP^{stop523}$ allele in which PGK-Neo cassette is excised by FLP recombinase. **B.** Testing of FLP-mediated deletion by PCR with primers Z22 and Z39. This shows a 220 bp band for mouse numbers 81, 83 and 86. Numbers 81 to 88: Tail DNA of F1 offspring from the mating of targeted mice to FLP-deleter mice. M: DNA marker, N: H_2O as negative control.

The presence of FLP recombinase was also measured by PCR using primers B34 and B35 (Table 2.6) which give a 750 bp fragment (data not shown). After FLP-mediated recombination, it is advisable to get rid of FLP from the floxed mice, as homozygosity for FLP leads to mouse embryonic lethality (Baur *et al.*, 2000; Dymecki 1996). Therefore, the

F2 offspring positive for Flp-mediated deletion were further mated with wild-type C57BL/6 mice.

3.5.6 Towards the generation of a tissue-specific mouse mutant

In order to generate the tissue-specific *CBP* knock-out mice, I planned to use transgenic mice in which Cre expression is driven by the *CaMKII α* promoter. Expression of Cre starts postnatally in forebrain neurons, mostly in the hippocampus, the brain region essential for learning and memory, in the first 3-4 weeks when many neural circuits have already been formed (Burgin *et al.*, 1990). Therefore, recombination and CBP-deficiency will occur in forebrain neurons postnatally. This will avoid developmental defects caused during neurogenesis, as observed in conventional knock-outs of *CBP* (Tanaka *et al.*, 1997; Oike *et al.*, 1999a; Kung *et al.*, 2000). We will therefore be able to analyse the mutants in learning and memory paradigms. A transgenic mouse line has been obtained from Prof. Schütz (DKFZ, Heidelberg) which carries a bacterial artificial chromosome (BAC) covering the *CaMKII α* gene locus and which expresses Cre recombinase under the control of all regulatory elements of the *CaMKII α* gene. I am currently in the process of accumulating CBD2.floxed/CBD2.floxed mice (see section 3.5.5) for crossing to the *CaMKII α* -Cre mouse from Prof. Schütz.

3.5.7 Preliminary phenotypic analysis of *CBP*^{stop523} mice

Developmental defects of heterozygous *CBP*^{stop523} mice. After Cre-mediated excision, only one mouse had earlier been shown to be mosaic for *CBP*^{stop523} heterozygosity (Fig. 3.29). Presumably because of its mosaic status, this F1 mouse did not display any apparent phenotype. When further mating of this F1 mouse with wild-type C57BL/6 was

performed, only two of the 15 F2 progeny were confirmed to be heterozygous for CBD exon 2 (CBD2)-deletion by PCR (Fig. 3.30). Clearly, the heterozygous mutant mice were under-represented among weaned pups according to the Mendelian inheritance rule, and this situation had actually been observed in the mosaic status. Either a fraction of the heterozygous embryos probably died *in utero* or less CBD2 deletion was existing in mosaic F1 than non-CBD2 deletion. The heterozygous F2 CBP^{stop523} mice (CBD2^{+/-}) were profoundly smaller than the wild-type littermates (Fig. 3.32). One of them died at the age of 4 weeks.

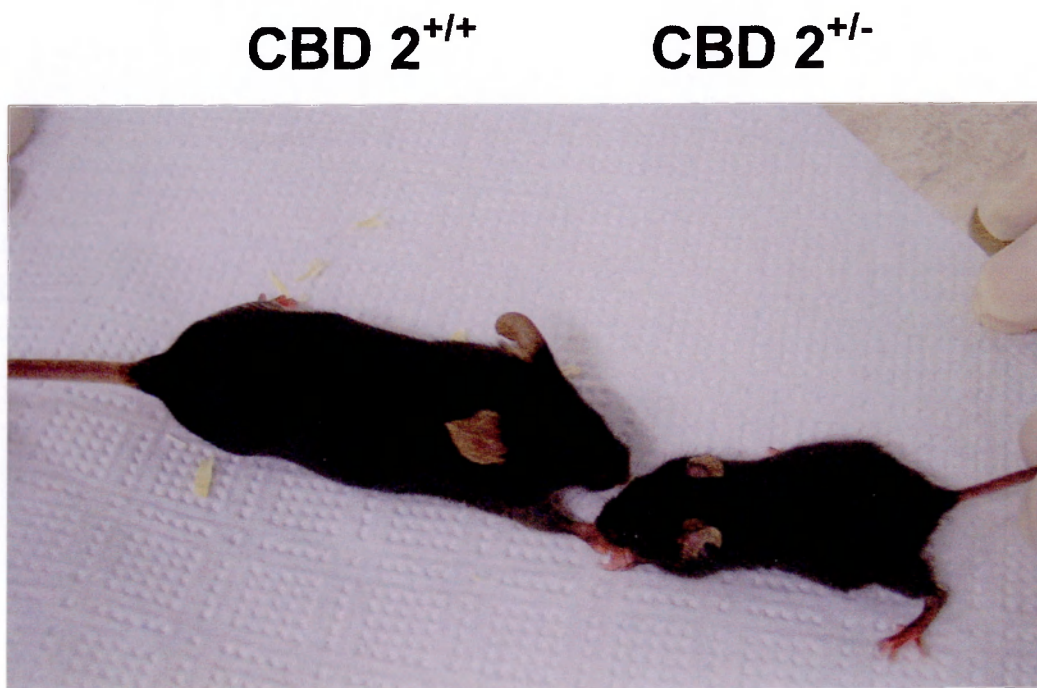


Fig.3.32 Comparison of heterozygous CBP^{stop523} mutant mouse (CBD2^{+/-}) with its wild-type littermate at the age of 5 weeks.

Investigation of embryos suggested that a fraction of heterozygous embryos died *in utero*. In total, 8 of 19 (42%) of analyzed embryos were shown to be CBD2^{+/-} by PCR (50% expected). Four of 10 embryos were CBD2^{+/-} in one mouse, and 2 of them died and started to be absorbed at E10.5; Four of 9 embryos at E8.5 in another mouse were

CBD2^{+/-}, no dead embryos were found. This indicates that the lethality in heterozygous CBD2 mutants occurs after E8.5.

3.6 Generation of CBP^{Tyr658Ala} knock-out mouse

3.6.1 Construction of the targeting vector CBP^{Tyr658Ala}

The targeting vector CBP^{Tyr658Ala} (Fig. 3.33 to 3.42) was constructed by introduction of a tyrosine to alanine substitution at residue 658 in CBD exon 5. The two loxP mutants lox66 and lox71 (see Fig. 1.8) flanked the sequence containing the wild-type exon 5, the inverted exon 5 with the Tyr658Ala point mutation, and the PGK-Neo cassette as a positive selection marker. According to BLAST searches, there is about 2.3 kb repetitive sequence in CBD intron 6 (referring to Fig. 3.37), therefore, only 1 kb of sequence is available for the right homology arm of construct CBP^{Tyr658Ala}. In order to increase the efficiency of the homologous recombination in surviving clones and to decrease random, non-homologous integration, the thymidine kinase (tk) gene was also included at the end of the shorter right homology arm as a negative selection marker. ES cells that do not contain any random integration do not contain the tk cassette and therefore have the ability to survive in the presence of 2 μ M Gancyclovir.

Generation of two loxP mutants

pBS-lox66 and pBS-lox71:

Primer Z24-Z25 were used to amplify the lox66 site with *Hind* III and *Eco*R I recognition sites at both ends, then subcloned into pBS II KS(-)/*Hind* III-*Eco*R I.

Primer Z26-Z27 were used to amplify the lox71 site with *Hind* III and *Eco*R I recognition sites at both ends, then subcloned into pBS II KS(-)/*Hind* III-*Eco*R I.

Symbols used for the construction:

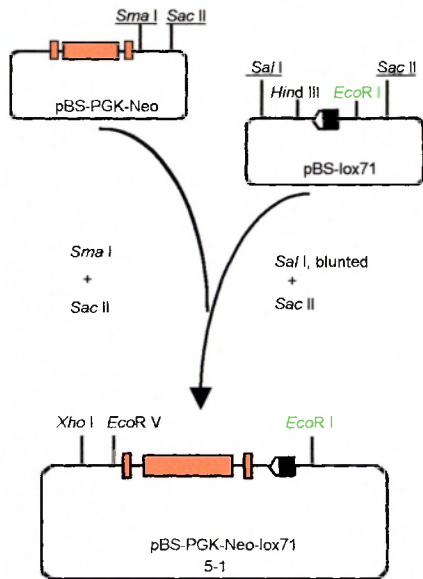
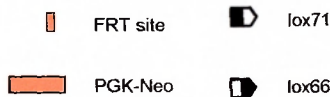
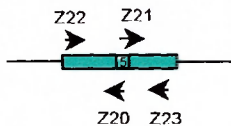


Fig. 3.33 Cloning of lox71 into pBS-PGK-Neo:
see construct CBP^{stop623} (Fig. 3-17 to 25)



- Two PCR fragments, Z22-Z20 and Z21-Z23, were amplified as template.
- Overlap PCR was performed with primer Z22-Z23 to amplify 1.0 kb genomic CBP including point mutation exon 5

* There is complementary overlapping between Z20 and Z21, both together introduced a new *Nru* I site following the point mutagenesis at aa658.

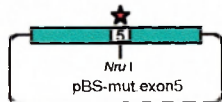


Fig. 3.34 Mutagenesis of a point mutation of CBP at aa658 in CBD exon 5 (CBDΔexon5).

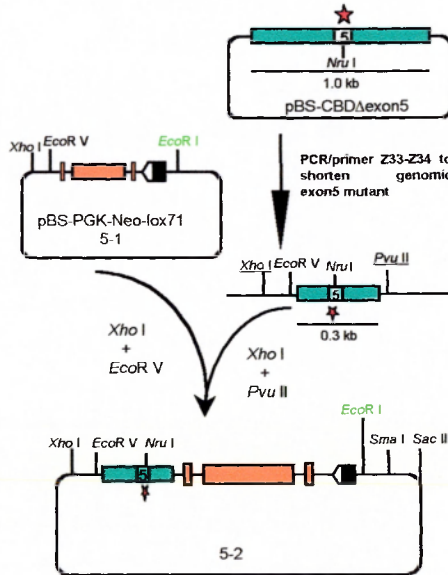
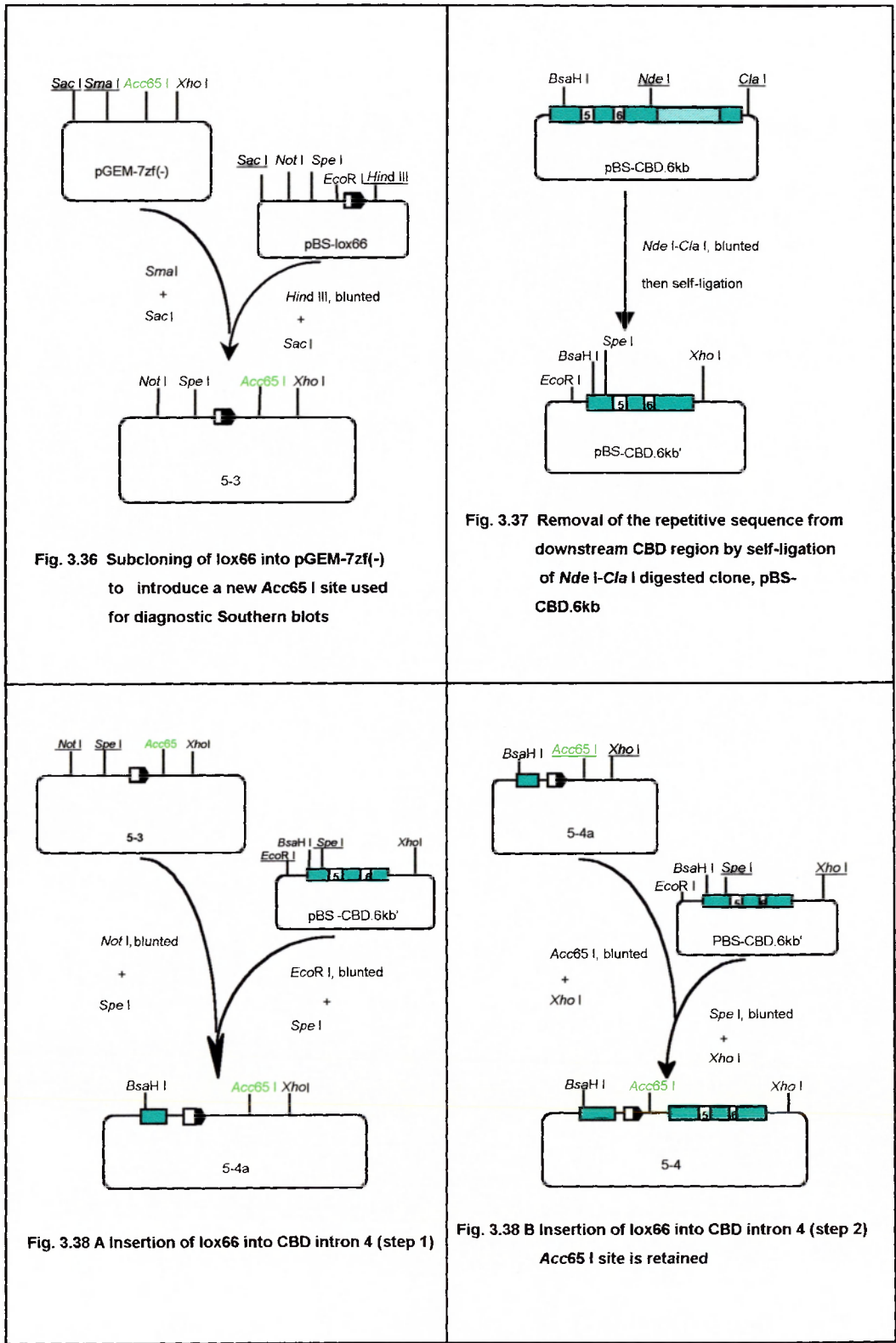
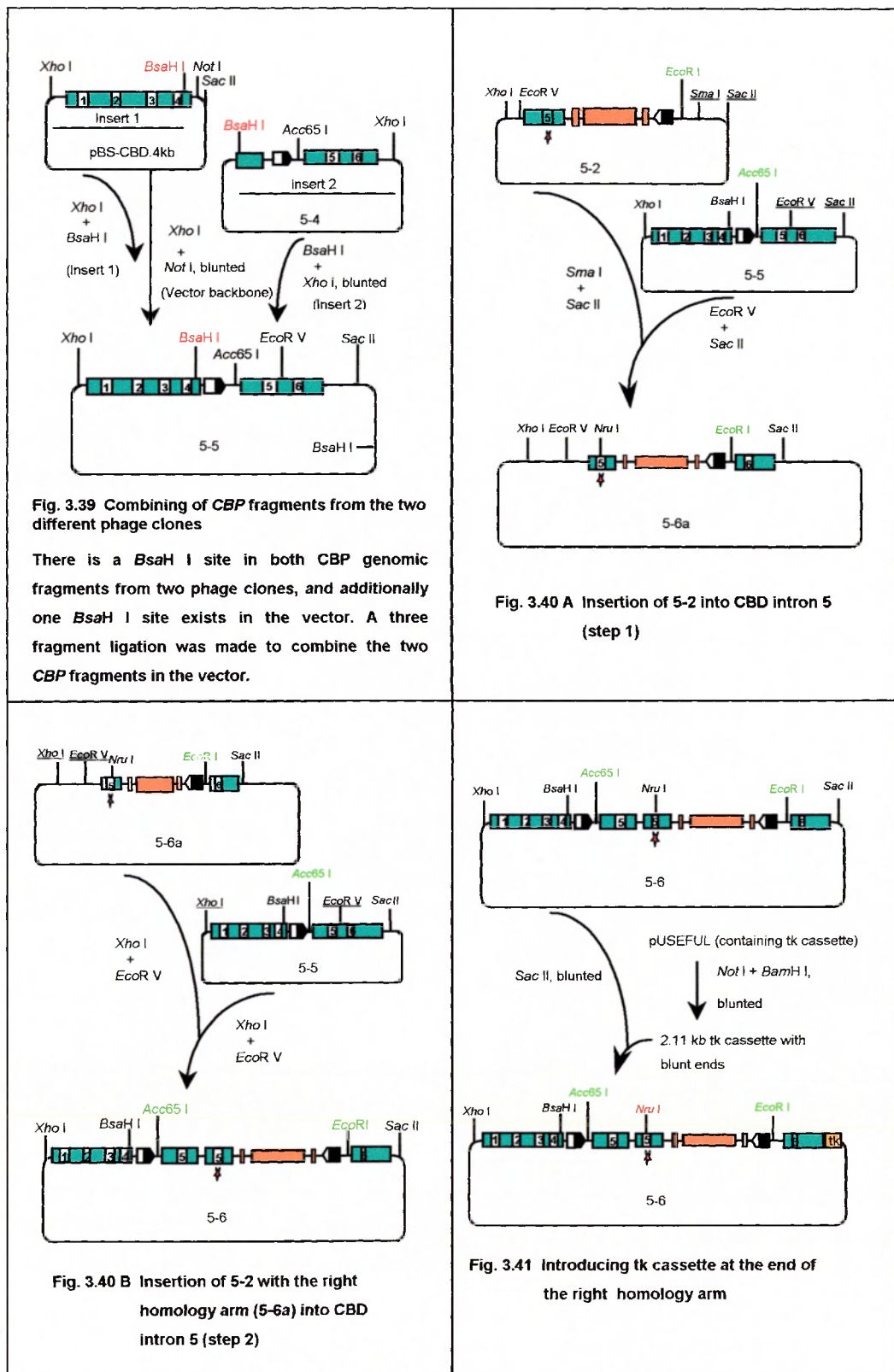


Fig. 3.35 Combining of PGK-Neo-lox71 with mutant CBDΔexon5





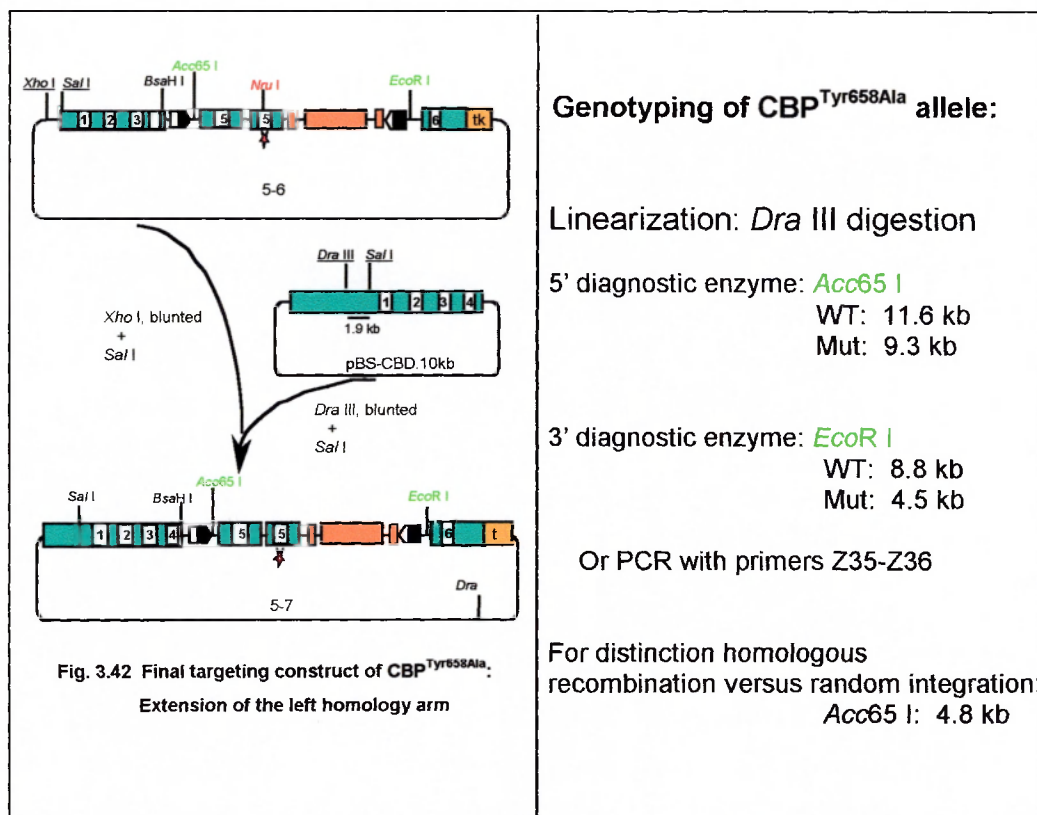


Fig. 3.33 to 3.42: Flow chart of construction of CBP^{Tyr658Ala}. CBD exon 5 and inverted CBD exon 5 with the point mutation were flanked by two mutated loxP sites lox66 and lox71 in opposite orientations. The PGK-Neo cassette flanked by two FRT sites in same direction was included between the two loxP sites as a positive selection marker. The tk cassette was included at the end of the right homology arm as a negative selection marker.

3.6.2 Screening for correct homologous recombinants of the targeted CBP^{Tyr658Ala} allele

100 µg of the targeting construct CBP^{Tyr658Ala} (Fig. 3.42) was linearized by *Dra*III digestion and checked by agarose gel electrophoresis. 20 µg of linearized plasmid was electroporated into mouse ES cells and grown in selection medium (G418, 200 µg/ml for entire period; and gancyclovir, 2 µM for the first two days). After nine days, resistant ES

colonies were visible, and 500 colonies were picked into 96-well feeder plates, then expanded on 96-well feeder plates for freezing and on gelatinized 96-well plates for Southern blot analysis. The screening strategy is shown in Fig. 3.43.

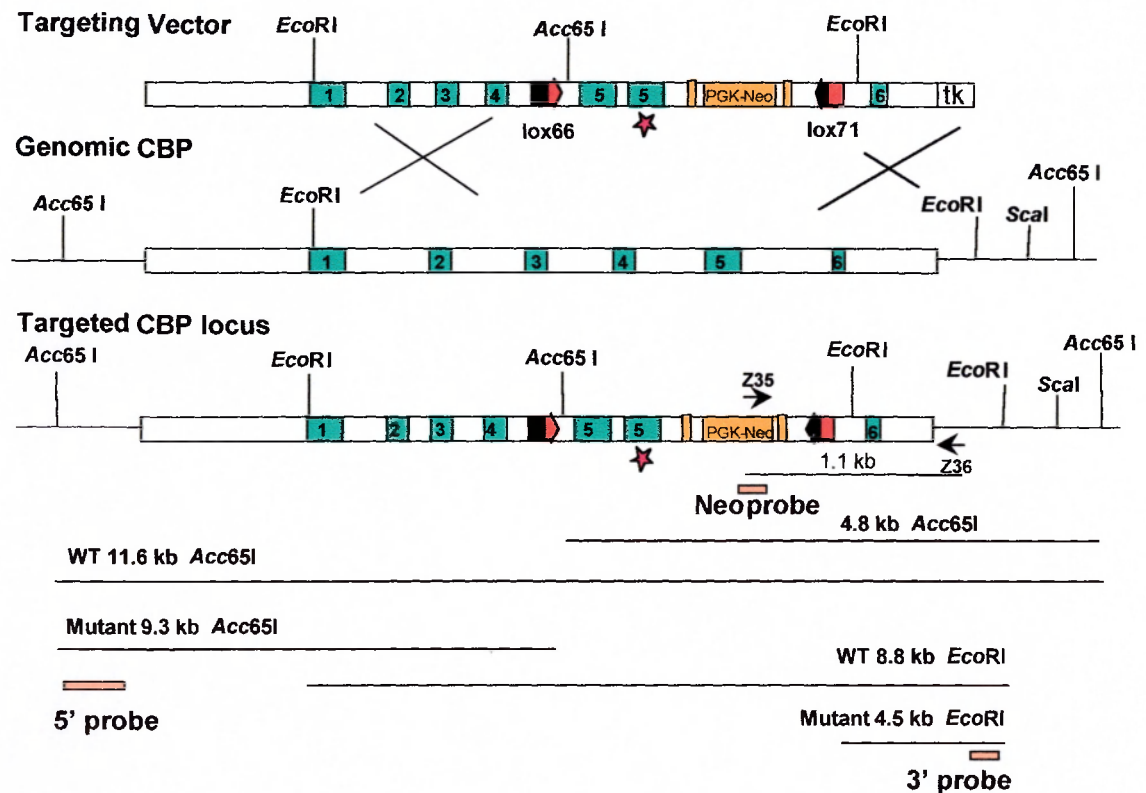


Fig. 3.43: Schematic representation of homologous recombination between the targeting construct $CBP^{Tyr658Ala}$ and *CBP* gene locus. Diagnostic probes and restriction fragments are also indicated: the 5' probe for correct homologous recombination of the left arm, the 3' probe for correct homologous recombination of the right arm, and the Neo probe for confirmation of homologous recombination and exclusion of additional random integration events. PCR using primers Z35 and Z36 (indicated with arrows) are performed instead of the 3' probe screening, a 1.1 kb band is expected for the right homologous arm.

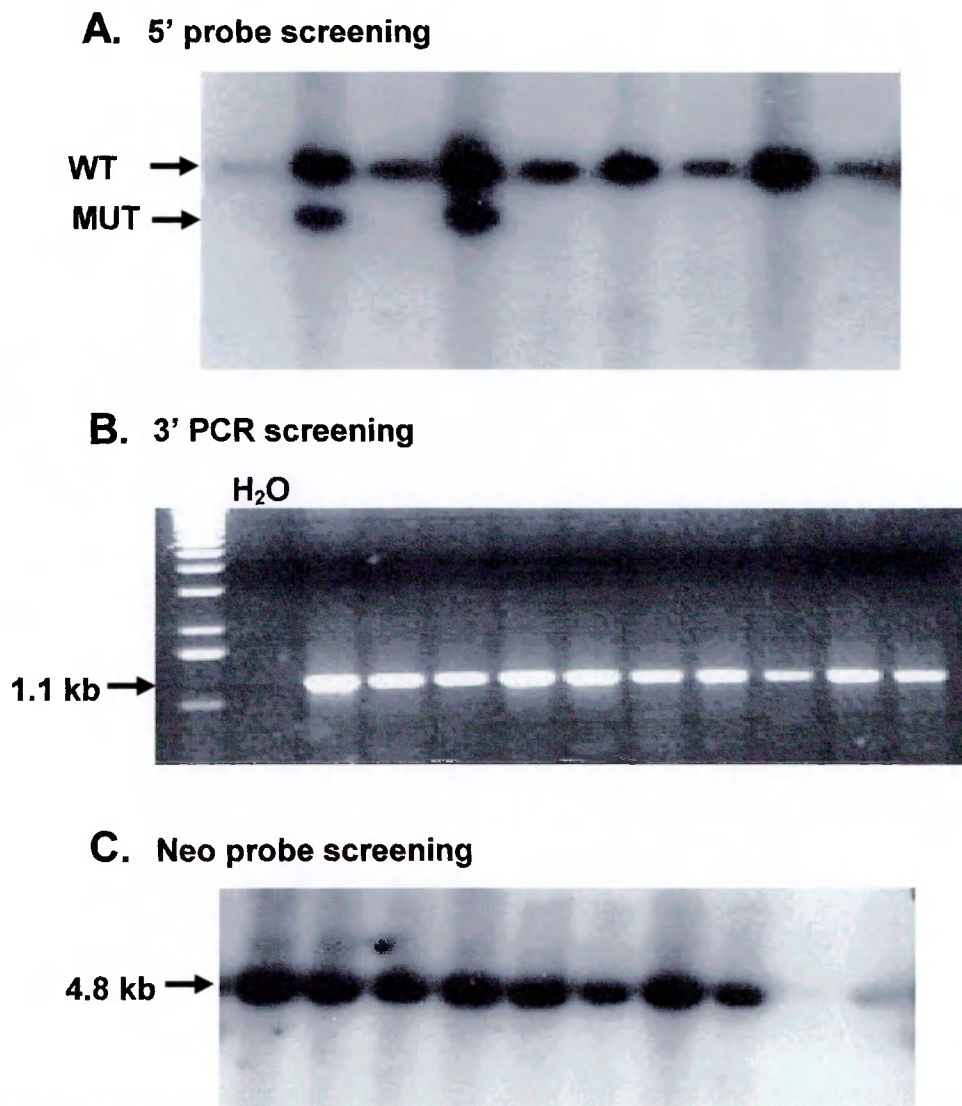


Fig. 3.44: Screening for correct homologous recombinants of the targeting construct $CBP^{Tyr658Ala}$.

A. Southern blot of *Acc65I* digested DNA from ES cell lines hybridized with the 5' probe was expected to show one 11.6 kb band for the wild-type and one 9.3 kb band for the targeted allele (Fig. 3.43). B. PCR using primers Z35-Z36. All 10 clones positive in the Southern screening using the 5' probe showed a positive band of 1.1 kb. H₂O as a negative control was included. C. Southern blot analysis of 10 clones with the mutated 5' and 3' fragments using the neo probe showed that these 10 clones gave one single band of 4.8 kb, thus excluding random integration.

Hybridization of the 5' probe to *Acc65I* digestions of ES cell DNA was used for the primary screen for homologous integration. Southern blot analysis (Fig. 3.44) showed that 17 of 500 (recombination efficiency: 3.4%) picked clones gave the expected pattern of one 11.6 kb wild-type and one 9.3 kb targeted band (see Fig. 3.43). Southern blot analysis of *EcoRI* digestions with the 3' probe failed, because the genomic DNA samples could not be fully digested with *EcoRI*. Verification was therefore carried out by PCR using forward primers Z35, located in the Neo gene, and reverse primer Z36, located just downstream of the right arm. Ten of 17 clones positive in the 5' screening had shown similarly intense hybridization signals for both targeted and wild-type bands using the 5' probe, and these 10 clones were therefore committed to further analysis by PCR. All 10 clones showed a positive band of 1.1 kb (Fig. 3.44), showing that correct homologous recombination occurred in the right arm. Furthermore, these 10 clones all showed one single band of the expected size of 4.8 kb (see Fig. 3.43 and 3.44) after *Acc65I* digestions and hybridization using the neo probe, indicating that no random integrations were detected in the 10 ES clones.

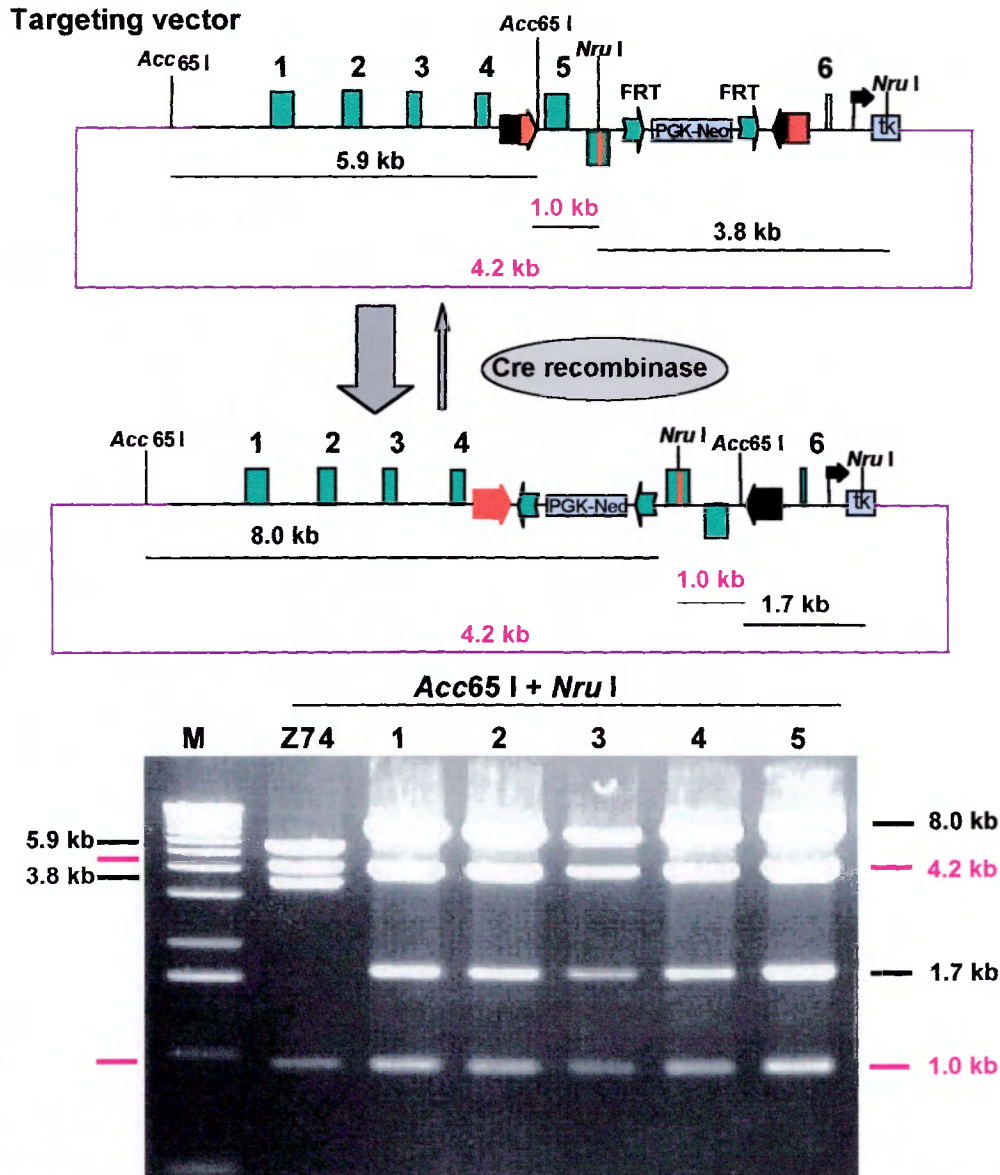
3.6.3 Examination of Cre-mediated inversion in *CBP^{Tyr658Ala}* allele

In order to test whether point mutation can be induced in the mouse genome by using Cre-mediated inversion and mutant loxP sites, I firstly tried to test it in bacteria and ES cells.

3.6.3.1 Examination of Cre-mediated inversion in bacteria

A Cre expressing plasmid, 705-Cre (carrying a chloramphenicol resistance gene), was transfected into DH5 α bacteria. To examine the lox66/lox71-mediated inversion, the

targeting construct $CBP^{Tyr658Ala}$ (Z74, containing the ampicillin resistance gene) was transformed into competent DH5 α (705-Cre) bacteria. Plasmid 705-Cre contains a temperature sensitive origin of replication that allows replication at 30°C but not at 37°C, where the plasmid will integrate into the genome. Five transformed colonies were inoculated into 2 ml of LB medium containing ampicillin (100 μ g/ml) and chloramphenicol (25 μ g/ml), and then incubated with shaking at 30°C overnight. Cre-mediated inversion was examined by restriction enzyme digestion of the DNA with *Acc65I* and *NruI*. All picked clones showed bands of 8.0, 4.2, 1.7 and 1.0 kb, and the control clone (clone Z74) showed bands of 5.9, 4.2, 3.8 and 1.0 kb. From the restriction map of the targeting construct (Fig. 3.45), it appeared that the two loxP mutant sites were able to mediate complete inversion. In contrast, the inverted sequence containing one wild-type loxP site and one double mutant loxP site was poorly recognized by Cre recombinase, because the digestion pattern of the initially transformed plasmid was not detected in the inverted Cre-expressing bacteria (Fig. 3.45), implying that the reverse reaction did not occur at a detectable frequency.



Z74: final construct of CBP^{Tyr658Ala}

1–5: five colonies of Z74 after Cre-mediated inversion

Fig. 3.45: Cre-mediated inversion in bacteria. The final clone of construct CBP^{Tyr658Ala} was transformed into *E. coli* DH5 α that expressed Cre recombinase. Digestion patterns of DNA before and after transformation showed that the two loxP mutant sites were able to mediate complete inversion. In contrast, the inverted sequence containing one wild-type loxP and one double mutant loxP sites was poorly recognized by Cre recombinase, because the digestion pattern of the initially transformed plasmid was not detected in the Cre-expressing bacteria, implying that the reverse reaction did not occur at a detectable frequency.

3.6.3.2 Examination of Cre-mediated inversion of $CBP^{Tyr658Ala}$ in ES cells

As a next step to verifying that tissue-specific generation of a $CBP^{Tyr658Ala}$ allele would be possible, induction of the Tyr658Ala substitution using Cre-mediated inversion was evaluated in one of 10 correct homologous recombinants (F3B12) by a transient transfection of Cre recombinase (plasmid pCre-pac) (Fig. 3.46).

Targeted $CBP^{Tyr658Ala}$ allele

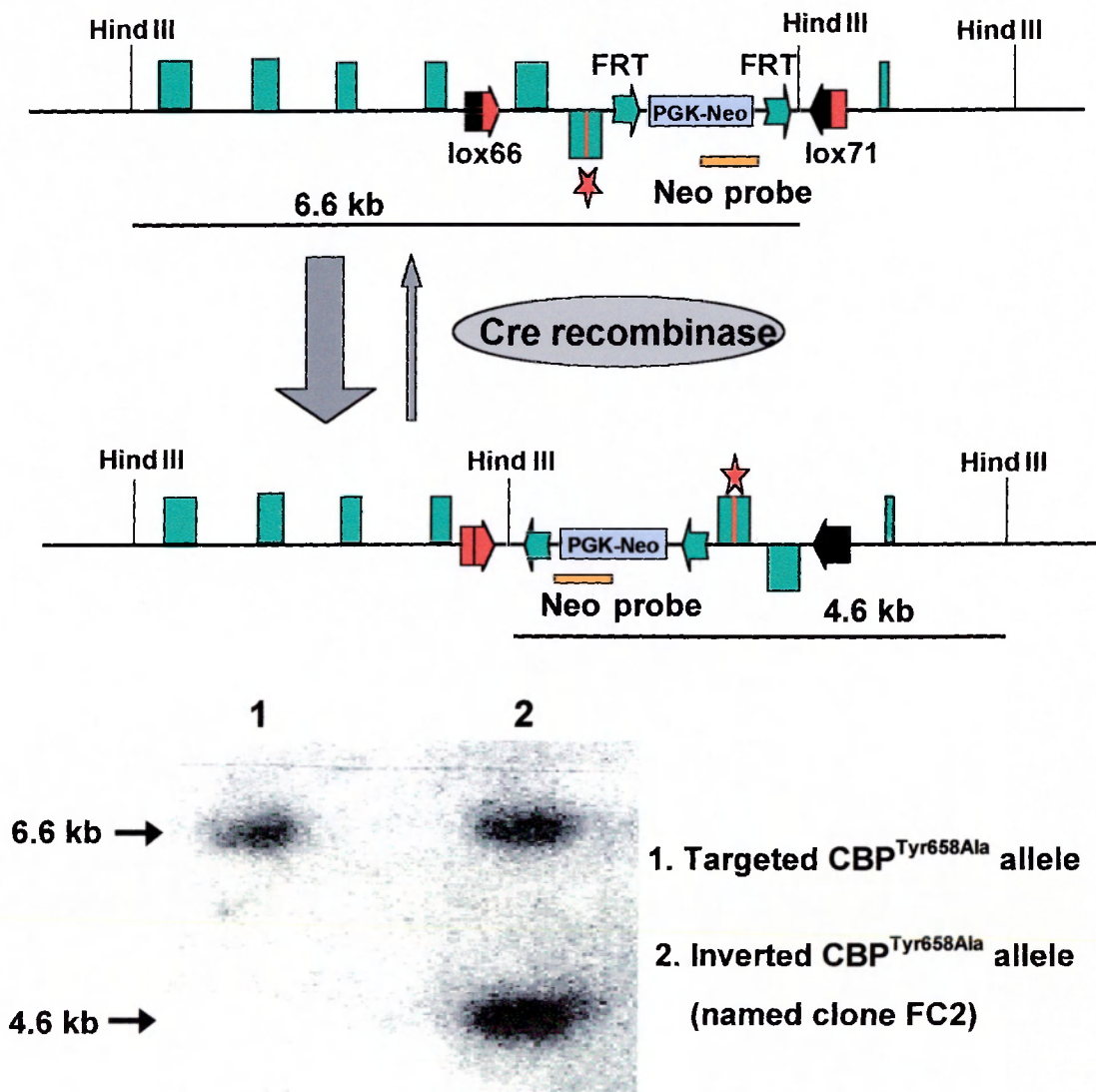


Fig. 3.46: Cre-mediated inversion in ES cells. The targeted $CBP^{Tyr658Ala}$ ES cell clone F3B12 was transfected with plasmid pCre-pac. Southern blot analysis of *Hind*III digested DNA using the Neo probe showed a 6.6 kb band for the original targeted allele and a 4.6 kb band for the inversion pattern of the targeted locus.

Southern blotting of *Hind*III digested DNA from transfected cells using the Neo probe was carried out as shown in Fig. 3.46. The original targeted cell clone F3B12 gave one 6.6 kb band. After transient transfection with Cre recombinase, one 6.6 kb band from the original targeted allele and one 4.6 kb band from the inversion were detected, thus, indicating that inversion can work in ES cells. The presence of a 6.6 kb band suggests that either the picked clone was contaminated with the original clone F3B12 or that inversion was incomplete.

3.6.4 Examination of splicing pattern of the inverted allele of the targeted **CBP^{Tyr658Ala}**

3.6.4.1 Excision of PGK-Neo cassette

To allow the analysis of the splicing pattern of the modified CBP locus, one of the ES cell clones containing the inversion (FC2) (see Fig. 3.46) was transiently transfected with the Flp-encoding plasmid pCAGGS-FLPe to achieve the removal of the PGK-Neo cassette.

Southern blotting using the Z33-Z34 PCR product (CBD Δ exon5, see Fig. 3.35) as a probe was carried out as shown in Fig. 3.47. If the PGK-Neo cassette is fully excised, one 2.6 kb band and one 6.1 kb wild-type band are expected. Because of the low efficiency of recombination catalysed by Flp, lack of excision and insufficient excision of PGK-Neo were also observed.

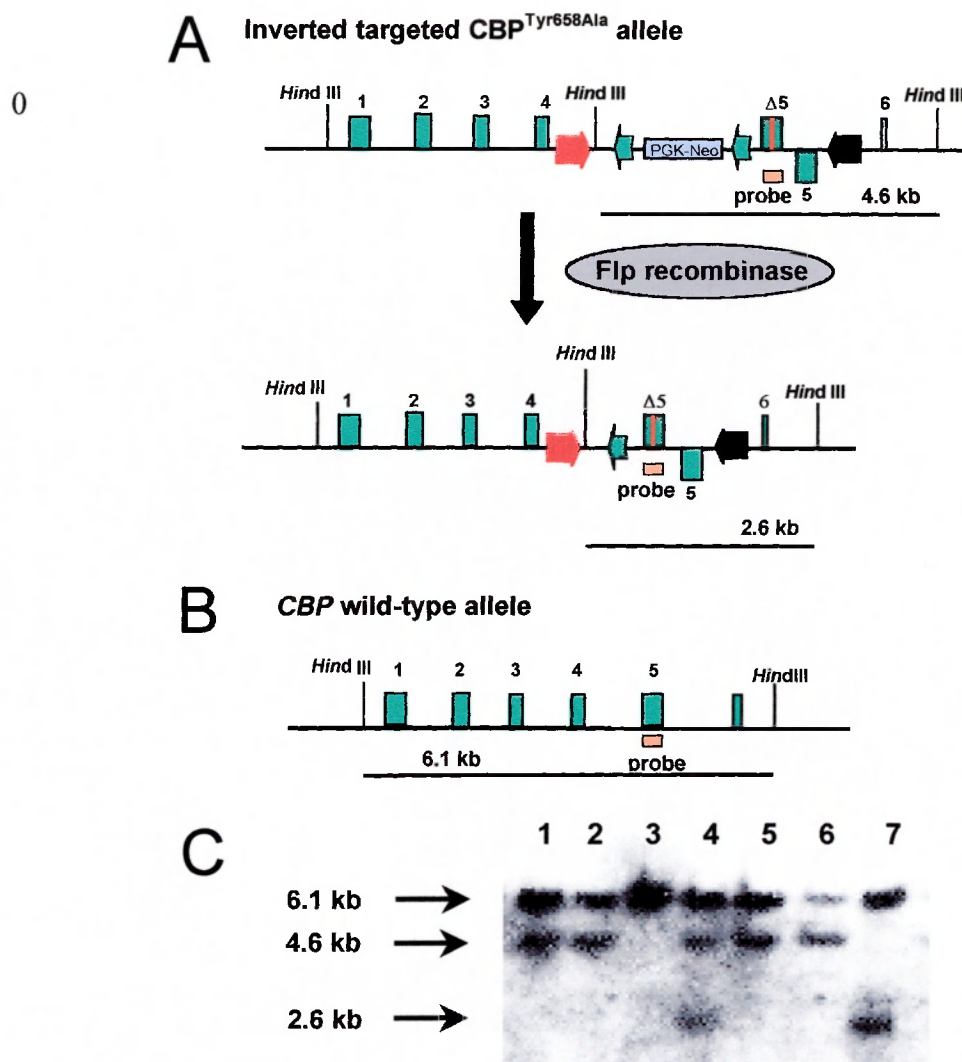


Fig. 3.47: Flp-mediated excision of the PGK-Neo cassette in the inverted targeted $CBP^{Tyr658Ala}$ allele. **A.** The ES cell line FC2 was transfected with pCAGGS-FLPe. **B.** CBP wild-type allele. **C.** Southern blot analysis using the Z33-Z34 PCR product as a probe showed a 4.6 kb band for the original inverted allele, a 2.6 kb band for the excision pattern of PGK-Neo and a 6.1 kb band for the wild-type allele. Lanes 1,2,5,6: ES cell lines in which PGK-Neo was not excised; lane 3: wild-type ES cells; lane 4: PGK-Neo was excised in some cells; lane 7: PGK-Neo was efficiently excised (named clone FC4).

3.6.4.2 Examination of splicing pattern of the inversion allele

RNA was then isolated from the “inversion⁺/neo⁻” ES cell clone (FC4 in Fig. 3.47). RT-PCR was performed with primers Z41 and Z42. The expected 460 bp fragment was obtained. Unfortunately, after digestion of the 460 bp band with *Nru* I, no further cutting was observed, indicating the lack of *Nru* I and, thus, the lack of mutated exon 5. Another two runs of PCR with two pairs of primers (Z41-Z43 and Z42-Z44, for the positions of all primers, see Table 2.6) were therefore carried out, showing that the splicing patterns at both 5' and 3' to the mutated exon 5 were wrong (Fig. 3.48).

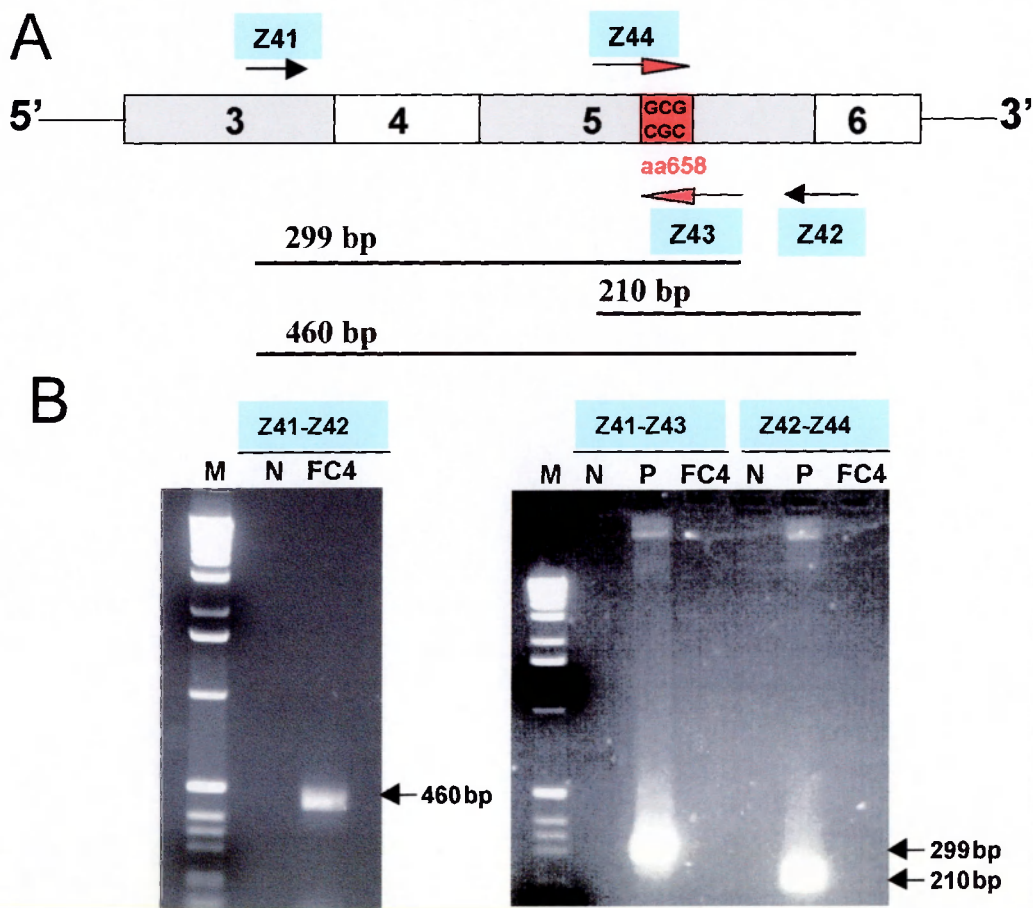


Fig. 3.48: Examination of splicing pattern of the inverted targeted $CBP^{Tyr658Ala}$ allele. **A.** Positions of primers Z41 to Z44 in cDNA sequence are labelled. The substitution of Ala658 is marked in red, both 3' ends of Z44 and Z43 complementary to this residue are also marked in red. Numbers 3 to 6 represent the CBD exons 3 to 6. **B.** RT-PCR using primers Z41-Z42 gave the expected 460 bp band. RT-PCR using two other pairs of primers (Z41-Z43 and Z42-Z44) revealed lack of correct splicing in FC4 cell clone. M: DNA marker X; N: H₂O as negative PCR

controls; P: Mutant CBPA₆₅₈ as positive PCR controls; FC4: an ES cell clone which is “inversion⁺/neo⁻” (referring to Fig. 3.47).

3.6.5 Mating for the germline transmission of the targeted CBP^{Tyr658Ala}

In order to obtain mice from the ES cell clones containing the targeted CBP^{Tyr658Ala} allele, three correct recombinant ES cell clones F2B3, F2H6 and F3B12 (obtained in Section 3.6.2) were selected for injection into C57BL/6 mouse derived blastocysts. The result of blastocyst injection of the targeted CBP^{Tyr658Ala} ES cell lines (in which WT CBP should be expressed) was summarized in Table 3.3.

Table 3.3 Summary of blastocyst injection of the targeted CBP^{Tyr658Ala} ES cells

Cell line	Number of		Number of chimeras		Germline transmission
	injected embryos	Number of pups born	(% of E14-derived coat)		
F2B3	143	16	2 (60, 80%)		Mating
F2H6	22	5	2 (30, 85%)		Mating
F3B12	354	29	4 (30, 50, 50, 70%)		Mating

Eight chimeras, which contained 30-85% of ES-derived coat were born. One, from ES cell line F3B12, died at the age of 6 weeks, and the others were mated with wild-type C57BL/6 mice for germline transmission. So far, no germline transmission of the targeted CBP^{Tyr658Ala} has been observed. This suggests that the targeted allele leads to dominant embryonic lethality. If the splicing in this line is incorrect, as it is in the ES lines containing the targeted construct (Fig. 3.48), then either truncated or other unknown CBP variants could be formed, and have dominant negative effects on viability of the mouse, as observed in the CBD2^{+/-} mouse line.

In order to obtain germline transmission, the matings are still continuing, but mouse strain CD1 is used for crossing to the chimeras instead of C57BL/6. If this is achieved, we could detect whether Cre-mediated inversion also works *in vivo* and furthermore, whether any CBP variants are actually formed by incorrect splicing.

3.7 Conclusions on generation of CBP mutant mice

Two constructs were generated to establish conditional *CBP* mutant mice, $CBP^{stop523}$ and $CBP^{Tyr658Ala}$ (referring to Sections 3.5.1 and 3.6.1). Both constructs were electroporated into ES cells, the correct homologous recombinants were obtained and then injected into blastocysts derived from C57BL/6 mice, which were then transferred into pseudopregnant mice. Several chimeras for both constructs were obtained. Construct $CBP^{stop523}$ was successfully transmitted in the germline, but $CBP^{Tyr658Ala}$ failed, possibly due to incorrect splicing resulting from the hairpin structure formed by the inverted sequences. Three mouse lines were generated from the construct $CBP^{stop523}$. Firstly, $CBD2^{+/-}$ has a general deletion of CBD exon 2 in all tissues. Preliminary results suggest that this mouse line shows severe developmental deficits. Secondly, $CBD2.floxed$ has the CBD exon 2 flanked by two loxP sites, and should behave like a wild-type mouse, but can be mated to different Cre-expressing mice to generate conditional mutant mice. Thirdly, $CBD2.floxed.neo$ is a mouse line in which the *Neo* gene is still present and can be used to test the possible interference of the PGK-Neo insertion on the cis-neighboring *CBP* gene, resulting in lower or null expression of *CBP*, i.e. hypomorph.

Chapter 4

Discussion

One of the hallmarks of memory consolidation in both vertebrates and invertebrates is the requirement for new protein synthesis. Long-term but not short-term memory requires new mRNA expression and new protein synthesis and is reflected in an altered expression level of specific proteins regulated by the cAMP second messenger pathway.

Transcription factor CREB is proposed to be a key factor to mediate memory consolidation, as both mice homozygous for a hypomorphic allele of the *CREB* gene and *Drosophila* overexpressing a dominant-negative form of CREB showed a reduced ability to form long-term memory (Bourtchuladze *et al.*, 1994; Yin *et al.*, 1994 and 1995). The switch from short- to long-term facilitation induced by behavioural sensitization in *Aplysia* involves CREB-like proteins, as the formation of long-term facilitation in *Aplysia* can be disrupted by drugs that interfere with transcription and translation, and by inhibiting cAMP-dependent gene expression (Dash *et al.*, 1996). However, CREB itself is not sufficient for transcriptional induction of targeted genes, as phospho-CREB does not appear to associate directly with factors of the basal transcription machinery as examined by an affinity selection assay. CREB needs the transcriptional cofactor CBP to interact with the basal transcription machinery to further regulate gene transcription.

CBP is a large nuclear phosphoprotein that was originally characterized as a cofactor for CREB, and itself is directly regulated by nuclear Ca^{2+} and Ca^{2+} /calmodulin kinase IV. Therefore, CBP is considered as an important site of regulation for Ca^{2+} -induced gene expression (Chawla *et al.*, 1998). However, CBP does not bind directly to DNA. Furthermore, CBP serves also as a cofactor for many other transcription factors,

transcriptional cofactors and kinases (for review see Janknecht and Hunter, 1996; Goodman and Smolik, 2000). To the present, very little knowledge has been gained on the function of CBP in memory consolidation. However, it has been found that *CBP* is mutated in the human Rubinstein-Taybi Syndrome (RTS) (Petrij *et al.*, 1995), a genetic disease characterized by multiple developmental defects and severe mental retardation. We therefore hypothesized that CBP might play a crucial role in the formation of long-term memory.

4.1 Expression pattern of CBP in adult mouse brain

Although the physiological role of CBP in transcriptional mechanisms has been the focus of many studies, very little is known about the expression and function of CBP in adult mouse brain. Therefore, the expression patterns of CBP and its related protein p300 were examined in adult mouse brain by *in situ* hybridisation. This showed that both CBP and p300 are present in most neuronal populations in mouse forebrain (Fig. 3.1), including the so-called limbic system, which is considered as central for many important brain functions, e.g. learning and memory, emotion, cognition and anxiety (Suzuki, 1996; Miller *et al.*, 1998). “Enhanced” *in situ* hybridization with DIG-labelled riboprobes revealed that CBP is not expressed ubiquitously in many brain areas, including the forebrain region. For example, CBP-negative cells were found in the dentate gyrus, cortical layer IV and even hippocampal CA1 and CA3 areas. Furthermore, it showed that there are differences in density of CBP expression between the two sides of the midaxis. This peculiar observation is in accordance with Strömberg’s report of CBP distribution in rat brain (Strömberg *et al.*, 1999).

Immunostaining showed that CBP protein was present in forebrain regions, e.g. intense signals were observed in hippocampal CA1, CA3, dentate gyrus and different layers of

cortex. However, CBP protein was not ubiquitously present, consistent with the expression patterns of CBP at mRNA level.

These results indicate that CBP might be involved in the regulation of gene activity in the adult brain.

4.2 Genomic organization of *CBP*

So far, detailed information on the *CBP* gene is only available from the human genome. The human *CBP* gene, which encodes a 7.3 kb transcript, appears to cover 159 kb genomic sequence (Chrivia *et al.*, 1993). The gene is localized on chromosome 16p13.3, and is transcribed from centromere to telomere. At least 14 exons were identified by PCR amplification of human genomic DNA (Giles *et al.*, 1997b). The genomic structure in CBD region is quite similar to mouse *CBP* characterized in this thesis. One splice donor site is located at nucleotide 798 shown to be near the site of one translocation breakpoint, and a deletion breakpoint in one RTS patient was found at this very same *CBP* splice donor site. Recently, more downstream exons of human *CBP* were well characterized by Petrij *et al.* (2000), showing that the CBP open reading frame is encoded by 31 exons in total that span 146 kb of genomic DNA from start (ATG) to stop (TAG).

Mouse *CBP* gene is located on chromosome 17 at the position of 11.0 cM from the centromere. A full-length cDNA clone has been sequenced and comprises 7,326 nucleotides. CBP is a large nuclear protein of 265 kDa and 2,441 amino acid residues. In order to generate *CBP* mutant mice, two independent phage clones were isolated, which encompass about 28 kb of genomic *CBP* sequence, including the CREB-binding domain (CBD). By comparing genomic and cDNA sequences (see Appendix II), 11 exons were characterized, covering about 2 kb of cDNA sequence. The CBD is encoded

by 6 exons, named CBD exons 1 to 6. There is no coding sequence detected in about 7.5 kb genomic sequence just upstream of the CBD exon 1. Comparing our results with the human genome sequence, and assuming that both mouse and human *CBP* genome share high similarity. This suggests that there may be two exons locating at least 7.5 kb upstream of CBD exon 1 (see Appendix II), which encode 1.3 kb of cDNA sequence. Furthermore, the downstream exons of mouse *CBP* encode another 4.0 kb of cDNA sequence could not be isolated from the phage libraries in this thesis.

The whole phage insert screened from one phage library was unable to be subcloned into plasmid vectors. It was first thought that the fragment was too big to be cloned. Actually that was not the case, as even the short fragment clone pBS-CBD.Nhe-Spe (Fig. 3.13) was difficult to be cloned. BLAST searches revealed 2.3 kb of highly repetitive sequences about 1 kb downstream of the CBD exon 5, which may be a reason for the failure to subclone the whole phage clone into the plasmid vector. This piece of sequence seems to cause problems in all bacterial strains used, always resulting in unstable clones or clones with deletions. The instability is probably due to repeats present in this piece of sequence, as it might allow ectopic recombination with similar sequences elsewhere in the genome, thus causing chromosomal rearrangements, indicating that this region might be involved in the breakpoints observed in RTS. However, all breakpoints reported so far are located in other regions of *CBP* gene (Petrij *et al.*, 1995; Borrow *et al.*, 1996; Sobulo *et al.*, 1997). Moreover, because of the possibility of recombination with ectopic sites, this highly repetitive sequence had to be avoided in the homology arm of targeting constructs. Therefore, only 1.0 kb could be used for the right homology arm in the construct $CBP^{Tyr658Ala}$. The tk cassette used in this construct as negative selection marker was aimed to increase the yield of clones with correct homologous recombination.

Remarkably, several discrepancies, A→T transversions, were found between the published cDNA sequence of *CBP* and the sequencing data that I obtained from clone pRc/RSV-mCBP.HA (see Appendix I). Thymidine (T) at nucleotide 2008, 2477, 2871 and 2932 of the mouse cDNA clone were detected instead of adenosine (A) in the published mouse cDNA sequence. Interestingly, all these discrepancies in mouse cDNA were replacement of the corresponding human cDNA sequence of *CBP* gene.

4.3 Functional analysis of CBP *in vitro*

As PKA activity is reported to be increased upon differentiation of F9 cells, and the CREB phosphorylated at residue Ser¹³³ is present in the nucleus only of differentiated F9 cells, but not in the nucleus of F9 embryonal carcinoma (EC) cells (Kingsley-Kallesen *et al.*, 1999), RA-induced, differentiated F9 cells were used for transfection experiments to investigate the properties of various *CBP* mutants. My results suggest that both CBD mutants (CBPA1a658 and CBP.CBDΔexon2-5) examined showed inhibitory effects on cAMP-mediated gene transcription. The repressive effects of CBPA1a658 as compared to the wild-type CBP seem to be dose-dependent. As F9 cells are rather sensitive to small variations in cell culture conditions, transfection reagents and plasmid purity, the *in vitro* analysis of CBP mutants will have to be modified and extended to other cell lines, e.g. HEK293. Nevertheless, our results clearly indicate that both CBD mutants interfered with cAMP signalling pathway and that the inhibitory effects of CBPA1a658 are dose-dependent.

I also measured the effects of the CBP mutants on dexamethasone-stimulated GRE-luciferase activity in transfection assays. Both CBPA1a658 and CBP.CBDΔexon2-5 showed profound activation of nuclear receptor signal transduction, revealing that both

mutants still retain other functions of CBP that are not related to the CBD, or at least the functions that require the N-terminal receptor interacting domain (RID).

All three RTS mutants showed strong inhibitory effects on the cAMP pathway. As major symptoms of RTS, such as developmental and mental retardation, may be related to CREB-mediated transcription, and RTS3 is similar to one of the knock-out mice (CBP^{stop523}), it is evident that the conditional mouse model for RTS might be a very useful tool to understand the complex mechanisms of the syndrome.

4.4 Cre-mediated inversion by using mutated Cre/loxP system

So far, inversion induced in a tissue-specific manner using a mutated Cre/loxP system (Albert *et al.*, 1995) has not been reported. For the construct CBP^{Tyr658Ala}, the inversion of the point mutated CBD exon 5 was tested in bacteria and ES cells. Both experiments showed that the targeted allele was inverted once. Cre recombinase efficiently recognized the lox66 and lox71 sites (forward reaction), and after inversion, the double mutant loxP and wild-type loxP sites were poorly recognized by Cre recombinase (reverse reaction). Cre-mediated recombination has been shown to be 4-fold to 7-fold more efficient between lox66 and lox71 than between double mutated loxP and wild-type loxP in tobacco protoplasts (Albert *et al.*, 1995). In our result, restriction digestion did not detect any of the original construct when Cre recombinase-mediated inversion was tested in bacteria (Fig. 3.45), and positive bands for the inversion were observed in the ES cells by Southern blot (Fig. 3.46). These observations suggest that the difference in recombination efficiency between forward and reverse reactions is much higher in bacteria and ES cells than in plants. However, it was not possible to evaluate the efficiency of Cre-mediated inversion quantitatively with the methods applied.

Nevertheless, our results suggest that the point mutation in CBD can be induced in mice using the mutated Cre/loxP system.

4.5 Generation of *CBP* knock-out mice

The functions of CBP and its closely related protein p300 have recently been investigated by gene targeting in mouse (Tanaka *et al.*, 1997; Yao *et al.*, 1998; Oike *et al.*, 1999; Kung *et al.*, 2000). Mice lacking both alleles of *CBP* die *in utero* between E8.5 to E10.5, mice double-heterozygous for *CBP* and *p300* have a comparable phenotype to that of *CBP* null mutants, and mice heterozygous for *CBP* display skeletal defects in the limbs similar to those observed in the human Rubinstein-Taybi syndrome (RTS) which is caused by mutations in the human *CBP* gene (Petrij *et al.*, 1995).

CBP-deficient mice will serve as a very useful tool to elucidate the physiological functions of CBP and to understand the complex mechanisms underlying RTS. The link between CBP and RTS symptoms was suggested by the interaction of CBP with multiple transcription factors. The molecular mechanisms of developmental and mental retardation of RTS still remain unclear. However, recruitment of CBP is sufficient for CREB-mediated gene activation (Cardinaux *et al.*, 2000; Shaywitz *et al.*, 2000), and CREB has been implicated in many forms of long-term synaptic plasticity and shown to be critical for memory consolidation. Thus, the mental retardation of RTS caused by mutations in *CBP* might be due to the impairment of CREB-mediated gene expression in central nervous system. It is evident that the conditional *CBP* knock-out mice will be a powerful tool to pinpoint the mechanisms underlying the multiple congenital malformations and mental retardation syndrome.

Cre-expressing mice are the other class of mouse lines used in the Cre/loxP system. Cre recombinase is expressed under the control of different regulatory sequences that can

drive Cre recombinase expression in specific tissues or cells and/or at specific developmental stage.

CBP is widely expressed in mouse forebrain areas, including the limbic system which is considered to be related with many central functions of brain, such as learning and memory. CaMKII α -Cre mice can now be used to generate a CBP deficiency restricted mostly to the hippocampus, cortex and thalamus. This can be a good model to analyze the function of CBP on learning and memory.

4.5.1 CBP^{stop523} knock-out mice

CBP^{stop523} is a deletion mutant derived from a construct in which the CBD exon 2 is flanked by two loxP sites. The exon 2 can be excised in the presence of Cre recombinase, and a new stop codon is then introduced at position 523, leading to a truncated N-terminal CBP. Similar deletion mutations of *CBP* have been reported in RTS. Another mouse model for RTS containing a truncated CBP has recently been generated by gene trapping (Oike *et al.*, 1999).

Three mouse lines were generated from the first targeting construct CBP^{stop523}. One is the floxed mouse line containing the PGK-Neo cassette. Because of the possibility of hypomorphic effects (Artelt *et al.*, 1991; Fiering *et al.*, 1993; Rijli *et al.*, 1994; Meyers *et al.*, 1998), the interference of the *Neo* gene on *CBP* gene expression is being tested. Secondly, the CBD2^{+/-} mouse line was obtained by mating of the germline mice containing the targeted allele with a Cre deleter mouse line (Schwenk *et al.*, 1995) to excise the CBD exon 2. Because of the efficiency of Cre-mediated recombination, there is a mosaic situation of excision and non-excision of the CBD exon 2 in F1 mice, and the allele carrying the excision was transmitted to 4 of 24 F2 mice. Clearly, the heterozygous mutant mice were under-represented among weaned pups according to the

Mendelian inheritance rule. The result indicates that the general deletion of the CBD exon 2 surely causes severe developmental defects. It is also evident that the conditional Cre-mediated excision of the CBD exon 2 using tissue-specific expression of Cre recombinase should work in mouse as well. Furthermore, CBD2^{+/-} mice might serve as a model for RTS, and an useful tool for functional analysis of CBP *in vitro*, for example, overexpression of wild-type CBP into the cell lines from the mutant mice to see whether this can rescue some phenotypic changes. Thirdly, as floxed mouse line in Cre/loxP system should behave like the wild-type before crossing with Cre-expressing mouse lines, and as the *Neo* gene located in an intron of *CBP* may cause a hypomorphic allele of *CBP*, the PGK-Neo cassette had to be removed. The floxed mouse line was obtained by mating of the germline mice (CBD2.floxed.neo) with a Flp deleter mouse line (Dymecki, 1996). Finally, this mouse line can be mated with different Cre-expressing mouse lines to generate tissue-specific *CBP* deletions.

The floxed mouse line, which carries the CBP^{stop523} allele and lacks the PGK-Neo cassette, is now in the process of being crossed to CaMKII α Cre-mice, in which Cre expression is driven by the CaMKII α promotor. Expression of Cre starts postnatally in most of the forebrain neurons, including the hippocampus, the brain region essential for learning and memory, in the first 3-4 weeks when many neural circuits have already been formed (Burgin *et al.*, 1990). Therefore, recombination, and hence CBP deficiency, will occur in the forebrain neurons postnatally. This should avoid developmental defects caused during neurogenesis as observed in conventional knock-outs (Tanaka *et al.*, 1997; Oike *et al.*, 1999a; Kung *et al.*, 2000). Certainly, this conditional knock-out mouse will be a useful tool to understand the functions of CBP on learning and memory.

4.5.2 CBP^{Tyr658Ala} knock-out mice

To elucidate the functions of CBP on CREB-mediated gene transcription, another CBP mutant mouse was proposed, in which a tyrosine to alanine substitution at amino acid residue 658 would be introduced by Cre-mediated inversion using the mutated Cre/loxP system (Albert *et al.*, 1995). Our results showed that longer inverted repeats were more difficult to clone into *E. coli*; a 300 bp repeat could be cloned, but a 780 bp repeat could not. After construction of the targeting vector and electroporation into the E14 cells, the correct homologous recombinants were verified by Southern blot and PCR, and injected into blastocysts isolated from C57BL/6 mice. Eight chimeras with ES-derived cell percentages ranging from 30-85% were obtained and mated with wild-type C57BL/6 mice for germline transmission at the age of about 8 weeks. Up to now, all pups (n = 57) derived from four of the chimeras showed a black colour characteristic of the C57BL/6 host. This suggested that the construct is not being transmitted, possibly because of strong interference of the modified allele on *CBP* gene expression. Two possibilities can be put forward: Due to the insertion of the *Neo* gene in a CBP intron, a hypomorphic *CBP* allele might have been generated. Secondly, might hairpin structures formed by the exon 5 and inverted exon 5 with the point mutation, might lead to aberrant splicing and thus to a variant protein, or to lack of protein expression. The later explanation might be favored, as RT-PCR experiments showed that the splicing pattern of the inverted allele was incorrect.

4.6 Preliminary phenotypic analysis of CBD 2^{+/-} mouse line

Mouse line CBD 2^{+/-} is a ubiquitous deletion mutant of CBD exon 2, obtained by crossing the targeted CBP^{stop523} mouse line with a Cre deleter mouse line (Schwenk *et al.*, 1995). Two of 17 F1 offspring were confirmed as positive for Cre-mediated

deletion. Neither showed any apparent phenotypes, and both had normal fertility. They were further mated with wild-type C57BL/6 to get heterozygous mice (CBD2^{+/-}). Among 24 F2 offspring, there were only 4 mice confirmed as heterozygous for the targeted allele (CBD 2^{+/-}). Such a reduced number of pups suggested that CBD 2^{+/-} may lead to embryonic lethality to some extent. This was confirmed by isolation of the embryos at E8.5 and E10.5. Furthermore, one of the heterozygous F2 mice died at the age of two weeks postnatally, and another mouse did survive, but displayed a significantly smaller size in comparison to its wild-type littermates (Fig. 3.32). As this mouse appeared less and less healthy, it was committed at the age of two months to allow dissection of internal organs for further phenotypic analysis. Another two, up to now, do not show any health problem except for the obviously smaller size, but they were infertile, as they could not give birth to pups after mating for more than two months. So far, more F2 CBD2^{+/-} mice have been obtained when the F1 was mated to the CD1 mouse strain than to the C57BL/6 strain. Interestingly, most F2 mice are female. The reason for this is not clear to the present. In conclusion, much stronger phenotypes were observed, compared to the phenotype of CBP mutant mice generated by others (Tanaka *et al.*, 1997; Oike *et al.*, 1999; Kung *et al.*, 2000). This is possibly due to the presence of the N-terminal amino acid residues 1-522, which acts as in a dominant-negative manner. Further phenotypic analyses are required to characterize the mutant mice.

Future perspectives

- 1) As the splicing pattern of the CBP^{Tyr658Ala} construct was found to be incorrect likely owing to a hairpin structure formed by the inverted mutated CBD exon 5 and wild-type CBD exon 5, it is proposed that elimination of the hairpin structure by introduction of silent point mutations into the inverted mutated CBD exon 5 sequence without interfering with the final protein product. This will be done by overlap PCR and confirmed by subsequent sequencing. This new construct will be introduced in to ES cells, to generate finally the mutant mice with the point mutation.
- 2) Further phenotypic analyses of CBD2^{+/-} mouse line at behavioral level, e.g. Morris water maze, fear conditioning experiments etc., and at cellular level, histological, immunohistological and *in situ* hybridization techniques to characterize expression differences and downstream targeted genes; primary neuronal cultures to analyze intracellular signal transduction pathways, will be performed.
- 3) Phenotypic analyses of forebrain-deficient CBP^{stop523} mutant mice are also been performed at the behavioral and cellular level to underline the functions of CBP on learning and memory.
- 4) Phenotypic analyses of CBD2.floxed.neo mouse line are also considered to underline the possible hypomorphic effects caused by insertion of *Neo* gene into the intron.
- 5) Electrophysiological characteristics will be measured in slices from hippocampus and amygdala. The *in vivo* LTP/LTD recording from the dentate gyrus of freely moving mice is being established.

Appendix I cDNA sequence of mouse CBP

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1 ATGGCCGAGA ACTTGCTGGA CGGACCGCCC AACCCCAAAC GAGCCAAACT
51 CAGCTCGCCC GGCTTCTCCG CGAATGACAA CACAGATTTT GGATCATTGT
101 TTGACTTGGA AAATGACCTT CCTGATGAGC TGATCCCCAA TGGAGAATTA
151 AGCCTTTTAA ACAGTGGGAA CCTTGTTCCA GATGCTGCGT CCAAACATAA
201 ACAACTGTCA GAGCTTCTTA GAGGAGGCAG CGGCTCTAGC ATCAACCCAG
251 GGATAGGCAA TGTGAGTGCC AGCAGCCCTG TGCAACAGGG CCTTGGTGGC
301 CAGGCTCAGG GGCAGCCGAA CAGTACAAAC ATGGCCAGCT TAGGTGCCAT
351 GGGCAAGAGC CCTCTGAACC AAGGAGACTC ATCAACACCC AACCTGCCCC
401 AACAGGCAGC CAGCACCTCT GGGCCCACTC CCCCTGCCTC CCAAGCACTG
451 AATCCACAAG CACAAAAGCA AGTAGGGCTG GTGACCAGTA GTCCTGCCAC
501 ATCACAGACT GGACCTGGGA TCTGCATGAA TGCTAACTTC AACCAGACCC
551 ACCCAGGCCT TCTCAATAGT AACTCTGGCC ATAGCTTAAT GAATCAGGCT
601 CAACAAGGGC AAGCTCAAGT CATGAATGGA TCTCTTGGGG CTGCTGGAAG
651 AGGAAGGGGA GCTGGAATGC CCTACCCTGC TCCAGCCATG CAGGGGGCCA
701 CAAGCAGTGT GCTGGCGGAG ACCTTGACAC AGGTTTCCCC ACAAATGGCT
751 GGCCATGCTG GACTAAATAC AGCACAGGCA GGAGGCATGA CCAAGATGGG
801 AATGACTGGT ACCACAAGTC CATTTGGACA ACCCTTTAGT CAAACTGGAG
851 GGCAGCAGAT GGGAGCCACT GGAGTGAACC CCCAGTTAGC CAGCAAACAG
901 AGCATGGTCA ATAGTTTACC TGCTTTTCCT ACAGATATCA AGAATACTTC
951 AGTCACCACT GTGCCAAATA TGTCCCAGTT GCAAACATCA GTGGGAATTG
1001 TACCCACACA AGCAATTGCA ACAGGCCCCA CAGCAGACCC TGAAAAACGC
1051 AAAGTATGATC AGCAGCAGCT GGTTCCTACTG CTTTCATGCCC ACAAATGTCA
1101 GAGACGAGAG CAAGCAAATG GAGAGGTTTCG NGCCTGTTCT CTCCCACACT
1151 GTCGAACCAT GAAAAACGTT TTGAATCACA TGACACATTG TCAGGCTCCC
1201 AAAGCCTGCC AAGTTGCCCA TTGTGCATCT TCACGACAAA TCATCTCTCA
1251 TTGGAAGAAC TGCACACGAC ATGACTGTCC TGTTTGCCCTC CCTTTGAAAA
      ** CBD exon 1 (CBD 1)
1301 ATGCCAGTGA CAAGCGAAAC CAACAAACCA TCCTGGGATC TCCAGCTAGT

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*****EcoR* I

1351	<u>GGAATTCAAA</u>	ACACAATTGG	TTCTGTTGGT	GCAGGGCAAC	AGAATGCCAC
1401	TTCCTTAAGT	AACCCAAATC	CCATAGACCC	CAGTTCCATG	CAGCGGGCCT
1451	ATGCTGCTCT	AGGACTCCCC	TACATGAACC	AGCCTCAGAC	GCAGCTGCAG
1501	CCTCAGGTTT	CTGGCCAGCA	ACCAGCACAG	CCTCCAGCCC	ACCAGCAGAT
		<u>Avr IICBD 2</u>			
1551	GAGGACTCTC	AATGCCCTAG	GAAACAACCC	CATGAGTGTC	CCAGCAGGAG
					<i>Sap</i>
1601	GAATAACAAC	AGATCAACAG	CCACCAAAC	TGATTTTCTG	ATCAGCTCTT
	<u>I</u>		<u>CBD 3</u>		
1651	CCAACCTTCCT	TGGGGGGCTAC	CAATCCACTG	ATGAATGATG	GTTCAAACCT
1701	TGGTAACATT	GGAAGCCTCA	GCACGATACC	TACAGCAGCG	CCTCCTTCCA
1751	GCACTGGTGT	TCGAAAAGGC	TGGCATGAAC	ATGTGACTCA	GGACCTACGG
		<u>CBD 4</u>			
1801	AGTCATCTAG	TCCATAAACT	CGTTCAAGCC	ATCTTCCCAA	CTCCAGACCC
1851	TGCAGCTCTG	AAAGATCGCC	GCATGGAGAA	CCTGGTTGCC	TATGCTAAGA
				<u>CBD 5</u>	
1901	AAGTGGAGGG	AGACATGTAT	GAGTCTGCTA	ATAGCAGG	GA TGAATACTAT
		<u>***aa658</u>			
1951	CATTTATTAG	CAGAGAAAAT	CTATAAAATA	CAAAAAGAAC	TAGAAGAAAA
	<u>*T</u>				
2001	GCGGAGGACA	CGTTTACATA	AGCAAGGCAT	CCTGGGTAAC	CAGCCAGCTT
2051	TACCAGCTTC	TGGGGCTCAG	CCCCCTGTGA	TTCCACCAGC	CCAGTCTGTA
		<u>CBD 6</u>			
2101	AGACCTCCAA	ATGGGCCCCCT	GCCTTTGCCA	GTGAATCGCA	TGCAGGTTTC
	<u>CBD 7</u>				
2151	TCAAGGGATG	AATTCATTTA	ACCCAATGTC	CCTGGGAAAC	GTCCAGTTGC
2201	CACAGGCACC	CATGGGACCT	CGTGCAGCCT	CCCCTATGAA	CCACTCTGTG
				<u>CBD 8</u>	
2251	CAGATGAACA	GCATGGCCTC	AGTTCCGGGT	ATGGCCATTT	CTCCTTCACG
2301	GATGCCTCAG	CCTCCAAATA	TGATGGGCAC	TCATGCCAAC	AACATTATGG
2351	CCCAGGCACC	TACTCAGAAC	CAGTTTCTGC	CACAGAACCA	GTTTCCATCA
2401	TCCAGTGGGG	CAATGAGTGT	GAACAGTGTG	GGCATGGGGC	AACCAGCAGC
		<u>CBD 9</u>	<u>*T</u>		
2451	CCAGGCAGGT	GTTTCACAGG	GTCAGGAACC	TGGAGCTGCT	CTCCCTAACC
2501	CTCTGAACAT	GCTGGCACCC	CAGGCCAGCC	AGCTGCCTTG	CCCACCAAGT
2551	ACACAGTCAC	CATTGCACCC	GAATCCACCT	CCTGCTTCCA	CAGCTGCTGG
2601	CATGCCCTCT	CTCCAACATC	CAACGGCACC	AGGAATGACC	CCTCCTCAGC
2651	CAGCAGCTCC	CACTCAGCCA	TCTACTCCTG	TGTCATCTGG	GCAGACTCCT
2701	ACCCCAACTC	CTGGCTCAGT	GCCCAGCGCT	GCCCAAACAC	AGAGTACCCC
2751	TACAGTCCAG	GCAGCAGCAC	AGGCTCAGGT	GACTCCACAG	CCTCAGACCC

4301 ATCTGGACAG TATTCATTTT TCCGGCCCC GCTGCCTCCG GACAGCTGTT
 4351 TACCATGAGA TCCTCATCGG ATATCTCGAG TATGTGAAGA AATTGGTGTA
 4401 TGTGACAGCA CATATTTGGG CCTGTCCCC AAGTGAAGGA GATGACTATA
 4451 TCTTTCATTG CCACCCCCCT GACCAGAAAA TCCCCAAACC AAAACGACTA
 4501 CAGGAGTGGT ACAAGAAGAT GCTGGACAAG GCGTTTGCG AGAGGATCAT
 4551 TAACGACTAT AAGGACATCT TCAAACAAGC GAACGAAGAC AGGCTCACGA
 4601 GTGCCAAGGA GTTGCCCTAT TTTGAAGGAG ATTTCTGGCC TAATGTGTTG
 4651 GAAGAAAGCA TTAAGGAACT AGAACAAGAA GAAGAAGAAA GGAAAAAAGA
 4701 AGAGAGTACT GCAGCGAGTG AGACTCCTGA GGGCAGTCAG GGTGACAGCA
 4751 AAAATGCGAA GAAAAAGAAC AACAAGAAGA CCAACAAAAA CAAAAGCAGC
 4801 ATTAGCCGCG CCAACAAGAA GAAGCCCAGC ATGCCCAATG TTTCCAACGA
 4851 CCTGTGCGAG AAGCTGTATG CCACCATGGA GAAGCACAAG GAGGTATTCT
 4901 TTGTGATTCA TCTGCATGCT GGGCCTGTTA TCAGCACTCA GCCCCCATC
 4951 GTGGACCCTG ATCCTCTGCT TAGCTGTGAC CTCATGGATG GGCGAGATGC
 5001 CTTCTCACC CTGGCCAGAG ACAAGCACTG GGAATTCTCT TCCTTACGCC
 5051 GCTCCAAATG GTCCACTCTG TGCATGCTGG TGGAGCTGCA CACACAGGGC
 5101 CAGGACCGCT TTGTTTATAC CTGCAATGAG TGCAAACACC ATGTGGAAAC
 5151 ACGCTGGCAC TGCCTGTGT GTGAGGACTA TGACCTTTGT ATCAATTGCT
 5201 ACAACACAAA GAGCCACACC CATAAGATGG TGAAGTGGGG GCTAGGCCCTA
 5251 GATGATGAGG GCAGCAGTCA GGGTGAGCCA CAGTCCAAGA GCCCCAGGA
 5301 ATCCCGCGT CTCAGCATCC AGCGCTGCAT CCAGTCCCTG GTGCATGCCT
 5351 GCCAGTGTCG CAATGCCAAC TGCTCACTGC CGTCTTGCCA GAAGATGAAG
 5401 CGAGTCGTGC AGCACACCAA GGGCTGCAAG CGCAAGACTA ATGGAGGATG
 5451 CCCAGTGTGC AAGCAGCTCA TTGCTCTTTG CTGCTACCAC GCCAAACACT
 5501 GCCAAGAAAA TAAATGCCCT GTGCCCTTCT GCCTCAACAT CAAACATAAC
 5551 GTCCGCCAGC AGCAGATCCA GCACTGCCTG CAGCAGGCTC AGCTCATGCG
 5601 CCGGCGAATG GCAACCATGA ACACCCGCAA TGTGCCTCAG CAGAGTTTGC
 5651 CTTCTCTAC CTCAGACCA CCCGGGACTC CTACACAGCA GCCCAGCACA
 5701 CCCCAAACAC CACAGCCCC AGCCCAGCCT CAGCCTTCAC CTGTTAACAT

5751 GTCACCAGCA GGCTTCCCTA ATGTAGCCCG GACTCAGCCC CCAACAATAG
 5801 TGTCTGCTGG GAAGCCTACC AACCAGGTGC CAGCTCCCCC ACCCCCTGCC
 5851 CAGCCCCCAC CTGCAGCAGT AGAAGCAGCC CGGCAAATTG AACGTGAGGC
 5901 CCAGCAGCAG CAGCACCTAT ACCGAGCAAA CATCAACAAT GGCATGCCCC
 5951 CAGGACGTGA CGGTATGGGG ACCCCAGGAA GCCAAATGAC TCCTGTGGGC
 6001 CTGAATGTGC CCCGTCCCAA CCAAGTCAGT GGGCCTGTCA TGTCTAGTAT
 6051 GCCACCTGGG CAGTGGCAGC AGGCACCCAT CCCTCAGCAG CAGCCGATGC
 6101 CAGGCATGCC CAGGCCTGTA ATGTCCATGC AGGCCCAGGC AGCAGTGGCT
 6151 GGGCCACGGA TGCCCAATGT GCAGCCAAAC AGGAGCATCT CGCCAAGTGC
 6201 CCTGCAAGAC CTGCTACGGA CCCTAAAGTC ACCCAGCTCT CCTCAGCAGC
 6251 AGCAGCAGGT GCTGAACATC CTTAAATCAA ACCCACAGCT AATGGCAGCT
 6301 TTCATCAAAC AGCGCACAGC CAAGTATGTG GCCAATCAGC CTGGCATGCA
 6351 GCCCCAGCCC GGACTTCAAT CCCAGCCTGG TATGCAGCCC CAGCCTGGCA
 6401 TGCACCAGCA GCCTAGTTTG CAAAACCTGA ACGCAATGCA AGCTGGTGTG
 6451 CCACGGCCTG GTGTGCCTCC ACCACAACCA GCAATGGGAG GCCTGAATCC
 6501 CCAGGGACAA GCTCTGAACA TCATGAACCC AGGACACAAC CCCAACATGA
 6551 CAAACATGAA TCCACAGTAC CGAGAAATGG TGAGGAGACA GCTGCTACAG
 6601 CACCAGCAGC AGCAGCAGCA ACAGCAGCAG CAGCAGCAGC AACAAACAAA
 6651 TAGTGCCAGC TTGGCCGGGG GCATGGCGGG ACACAGCCAG TTCCAGCAGC
 6701 CACAAGGACC TGGAGGTTAT GCCCCAGCCA TGCAGCAGCA ACGCATGCAA
 6751 CAGCACCTCC CCATCCAGGG CAGCTCCATG GGCCAGATGG CTGCTCCAAT
 6801 GGGACAACTT GGCCAGATGG GGCAGCCTGG GCTAGGGGCA GACAGCACCC
 6851 CTAATATCCA GCAGGCCCTG CAGCAACGGA TTCTGCAGCA GCAGCAGATG
 6901 AAGCAACAAA TTGGGTCACC AGGCCAGCCG AACCCCATGA GCCCCAGCA
 6951 GCACATGCTC TCAGGACAGC CACAGGCCTC ACATCTCCCT GGCCAGCAGA
 7001 TCGCCACATC CCTTAGTAAC CAGGTGCGAT CTCCAGCCCC TGTGCAGTCT
 7051 CCACGGCCCC AATCCCAACC TCCACATTCC AGCCCGTCAC CACGGATACA
 7101 ACCCCAGCCT TCACCACACC ATGTTTCACC CCAGACTGGA ACCCCTCACC
 7151 CTGGACTCGC AGTCACCATG GCCAGCTCCA TGGATCAGGG ACACCTGGGG
 7201 AACCCTGAAC AGAGTGCAAT GCTCCCCCAG CTGAATACCC CCAACAGGAG

7251 CGCACTGTCC AGTGAAGTGT CCCTGGTTGG TGATACCACG GGAGACACAC

7301 TAGAAAAGTT TGTGGAGGGT TTGTAG

- * Instead of A, corresponding nucleotides (T) in human cDNA are detected in the original mouse cDNA clone pRC/RSV-mCBP.HA.
- ** Sequences marked in blue and black are 11 exons characterized in the isolated *CBP* genome (here named CBD exon 1 to 11).
- *** Coding sequences for tyrosine at amino acid 658.
- **** Underlined sequences are useful restriction sites for cloning, and mutagenesis of CBP for *in vitro* transfection.

Appendix II Genomic sequence of CBP in the region of the CBD exons

**BamH* I

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1  GGATCCCTGC TGTTTTTAGG CATCAGAGCA TTAAGAAGAG ATATGAATGC
51 CTCTTCCCAG TTTTAAGAAC CTTTGCTGTC ATTTTCAGAGG CTACATTGTG
101 GTCTTTAAAT ATGCCCCTCA TTTGCACATC TAGAAAATGC TCTTGGGTGG
151 GCATGCTCTA ATTGGAGGCA ATCCCTTTTT TAGTGATCTT TCAGGGTGAC
201 ACAGTCTTTC TCATCTTTCA CAATCTTACT TCTCCCCAGT GTTCTTGGTG
251 TTCTTAGAAC ATCTTCTTGA ATATCTTTAC CTGCCTGGTG AACATGTGCT
301 GCTTTCTTCC TGGCTGGCTG TACATGGCAA ATCACTCTAA TCTAAAAGTA
351 CTATGGAGTG CCATTGCTCT GTTCGATCTC TTATGAATCT AGCTTTTCTT
401 CCTGTGAGCT GATTCACACT GTATAGCATG CTTCAATTGGA AAAAACAGGA
451 TGTTGGGGCT TGATGTCTTC TTCCCTTACA GATGTACCAC CACAATTAAG
501 TTTCTTTGAA AACTTAGTTT CTTGAATCTT TTTGTGTATA TGTATTGGAG
551 GTGCTTGTGT GTGTGTATGT GTGTGTGTGT ACGGTTACTC ATTTGCCACA
601 GCGTGTGGTA TAGGTCAGAA ATGTTGGTTC TTACCCTTTA CTTTGTTTTA
651 AGGCTAGGTC TTTGTTGTTT ATGTGTCCTG TATATATAAG CTGGGTAGCC
701 TGTGAGTATC TAGAGATTGC TCTATCTCCA CCTGGCATTG GAACTGGGAT
751 TACATGCATG TTGCCATGGC TGGTTTTACA CAGGCTATGG AAATTTAAAC
801 TCAGGTCCTT AACTTGTCT GATAAGTACT TTAGCCTTCT TTGTAAGTCC
851 TAAATTTTTA ATATATTAAT TTTTTTTGAC ATTTCTTACA TAAAGTTCTG
901 GAATAGTGGG TCTTATTAGC CATGAATAAT GAGCTAGGAT CTCACTTTTA
951 AGTTTTAATT TTGGGATGTG ACTGAGGGAG CTTTGTAGCT TCGTATGTNC
1001 AAAGTGTAT GAAGATTGTA AAGCTTTGCA ATTTCTCTTT TTAAGAAATA
1051 ATGTGGGTTT TGTTCAAGAG AGAGGAAAAA AAGAAACATG GTTTGTTTTT
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1151 TTCTCAAGTG GTAAACCACA GTGTTTCTAT AACGTGAGCN GTTAGTGTGT
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 CBD 10
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 22601 CCAGGGAAAA GGTGATGAAG GAGTCAGGAT GGAAAATGAG CAAAATGCT
 22651 GTAGTTTGAG ACAA

- * Sequences marked in red are useful restriction sites for cloning, construction of the targeting vectors and diagnosis of correct homologous recombinants.
- ** Sequences marked in green encode part of exons of *CBP*, here named CBD exon 1 to 11.
- *** Sequences marked in blue are overlapped by two *CBP* fragments isolated from two phage libraries.

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